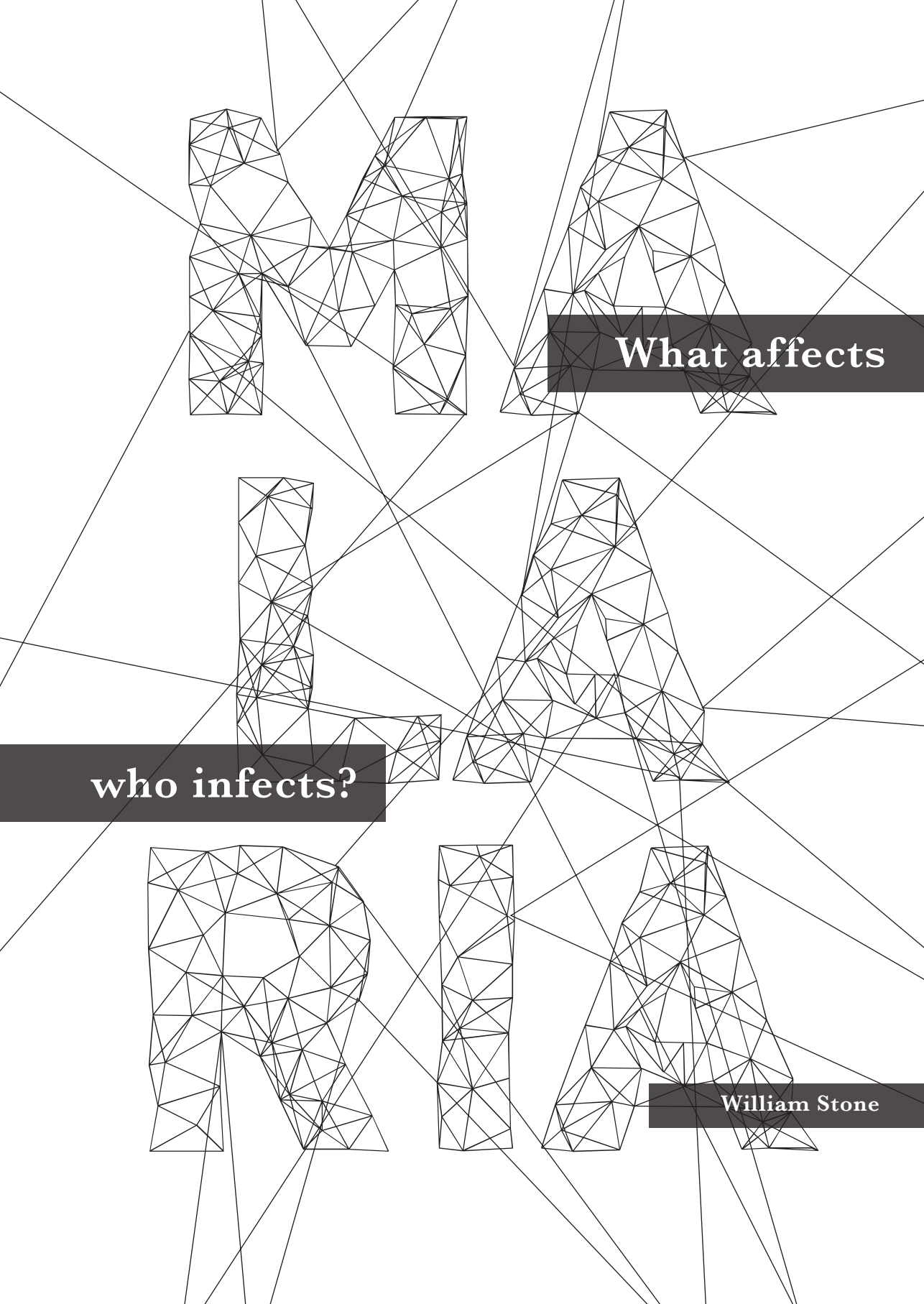
What affects
who infects?
William Stone

Colofon

ISBN: 978-94-92801-22-7

Cover design: proefschrift-aio.nl
Layout and print: proefschrift-aio.nl

Malaria: what affects who infects?

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op

donderdag 22 februari 2018,

om 16.30 uur precies

door

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geboren te Colchester, Verenigd Koninkrijk

op 26 juni 1988

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Doctoral thesis to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on

Thursday 22nd February 2018

at 16.30 hours

by

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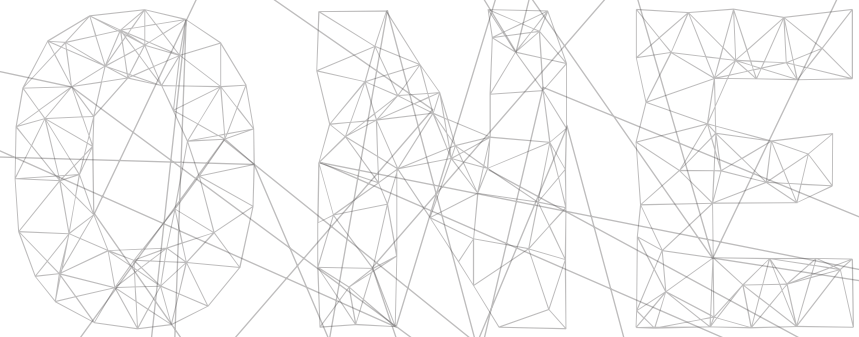
ONE

Chapter 1

Introduction

> "Well, in our country," said Alice, still panting a little, "you'd generally get to somewhere else—if you run very fast for a long time, as we've been doing."
>
> "A slow sort of country!" said the Queen. "Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"
>
> *— Lewis Carrol, Alice in Wonderland*

Malaria is conventionally controlled using drugs to kill the parasite that causes the disease, and insecticides and nets to prevent the parasites transmission to and from mosquitoes. One report estimates that in Africa 663 million cases of malaria were averted by control efforts between 2000 and 2015 [1]. It seems logical that gains of the same scale could continue if we were to apply the same control with greater coverage and intensity. Yet hundreds of millions are spent improving our control tools, seeking alternatives, and striving to better understand the malaria parasite and its vector. This begs questions: Is too much money funnelled into novel malaria research? Is malaria a problem of politics and misdirected funding?

Malaria control is currently effective because we so recently developed our latest controls that the parasite has not yet adapted to coexist with them. There is no denying this adaptation has begun though. Along the east African coast mosquitoes that once bit exclusively at night and indoors are now biting earlier and outdoors [2, 3]. Where mosquitoes with a strict preference for human blood have been wiped out by indoor insecticides, other less picky species are maintaining transmission by feeding on cattle as well as humans [4]. Perhaps more concerning are the changes that have been observed in mosquito and parasite physiology. Mosquito resistance to pyrethroid insecticides, which dominate the market because of their use in insecticide treated nets, is now widespread [5, 6]. In border regions of Thailand, Myanmar and Cambodia derivatives of Artemisinin, the latest and best antimalarial treatment, are becoming less effective against local parasite strains [7].

Our current arsenal of treatments and vector controls are variations on approaches used throughout history; avoid being bitten, kill the mosquito, and treat the infection. Though fundamentally these approaches have changed very little, the effectiveness of our tools current iterations is the product of an historic struggle against mosquito and parasite evolution. Resistance to quinine, used in various forms since the 17th century, has been repeatedly observed but develops slowly and sporadically [8], whereas Chloroquine and Sulphadoxine-pyrimethamine resistance emerged rapidly in the 1960's and 1990's respectively, and now appear fixed in many parasite populations [9]. The same pattern has been observed with insecticides, with mosquitoes becoming resistant in a matter of years to each generation of compounds since resistance to DDT emerged in the 1950's [6].

We have been running to stand still in the fight against malaria, and only in recent years do we appear to be outpacing it. By continuing to explore the biology and epidemiology of the parasite and its vector, scientists hope to reveal vulnerabilities to exploit with new drugs, vaccines, and insecticides. Without continued research, fundamental and applied, the potency of our current approaches will fall and the hard-won gains of the last two decades will slow and reverse. Investing to ensure these tools continue to be effective is, simply put – fine. With this approach we can hope to keep controlling malaria, and continue its decline to an extent. To get 'somewhere else' though, we must bring new weapons to the fight.

Plasmodium, parasitism, and transmission

The organisms that cause malaria in humans belong to the phylum Apicomplexa, and the genus *Plasmodium*. Apicomplexans evolved from a photosynthetic ancestor, diverging from colpodellids (marine predators) and chromerids (coral like photoautotrophs) by adopting obligate parasitism [10]. The phylum is divided into two classes; the Conoidasida, which are monoxenic parasites, meaning they require a single host, and the dixenic Aconoidasida, which need two hosts to complete their life cycle. *Plasmodium* belongs to the latter group, its life cycle being complete after development in both a female mosquito and a vertebrate host. The life stages emerging in each are essential, but the mosquito is the parasite's definitive host, in which it reaches sexual maturity.

The transmission event for *Plasmodium* is the mosquito bite. With the exception of *Toxorhynchites* mosquitoes, which form their own subfamily, females in all mosquito genera feed on vertebrate blood [11]. This is not because they require blood for survival (females get their basic nutrients from sugar and water, as males do) but because it is required for egg development. When mating, the female mosquito stores the male's sperm in a specialised structure called a spermathecae, so that afterwards she may produce eggs continually. Egg development (the gonotrophic cycle) takes three days, and as long as she feeds at least once in each cycle, an inseminated female mosquito may lay for the rest of her life. The malaria parasite appears to take advantage of this behaviour, so from our point of view it's easy to think of mosquitoes as the parasites tool; an insect used as a middle-man to get between infected and uninfected vertebrates. From a parasitological viewpoint, however, the vertebrate is the tool, serving only as a reservoir for parasites attempting the jump from one mosquito to the next. The term 'vector' then is epidemiological. A vector of human pathogens is an animal or object which carries a pathological agent and facilitates its transmission to humans. If a good vector is one regularly exposed to humans, increasing the chance of pathogen transmission, then blood-feeding (or haematophagous) vectors are exceptional. The parasite's aim, to gain entry to its secondary host, aligns precisely with that of the vector, which is to regularly feed. Because of the obvious efficiency of this vector-parasite system, blood-feeding animals have evolved to co-exist with an array of vertebrate viruses, microbes and parasites. These vary widely in medical importance, but

mosquitoes receive particular notoriety as sources of disease. In 2014 Bill Gates announced on his web-blog that mosquitoes were the 'deadliest of all animals', killing more people than humans and the next 13 most dangerous animals combined [12]. News of the Zika fever virus disease epidemic in South America has recently put *Aedes aegypti* and *Aedes albopictus* in the spotlight; mosquitoes otherwise infamous as vectors of the yellow fever, dengue fever, and chikungunya fever viruses. Medically important species in the genera *Culex* also transmit deadly arboviruses, including West Nile and Japanese encephalitis, as well as the worm *Wucheria bancrofti*, which causes lymphatic filariasis. Collectively, mosquito borne diseases are responsible for approximately 725,000 deaths every year [12]. Of these, more than half are the result of infection with malaria parasites [13]. Though *Culex* mosquitoes, as well as *Aedes*, *Mansonia,* and *Culiseta*, host a range of *Plasmodium* species, none develop fully or cause pathology in humans [14]. Only mosquitoes in the genus *Anopheles* transmit the parasites that cause human malaria, and of the 461 described *Anopheles* species, 41 are thought to be medically important, because they host species able to infect and develop in humans and because of their at least partial preference for our blood [15].

Five *Plasmodium* species cause malaria in humans: *Plasmodium malariae, Plasmodium ovale*, *Plasmodium knowlesi*, *Plasmodium vivax* and *Plasmodium falciparum*. *P. malariae* is widespread in areas of sub-Saharan Africa and the southwest Pacific (prevalence in these areas has exceeded 15-30%) but elsewhere the parasite occurs only at low frequency (1-2%) [16, 17]. *P. ovale* is uncommon, accounting for around 1-3% of reported cases in areas of West Africa and Southeast Asia [16], though molecular surveillance put its prevalence in some regions closer to 5-10% [16, 18]. *P. knowlesi*, the rarest of the five species, is a monkey parasite, and its infection of humans is a by-product of only partially overlapping biology with human infecting species, which makes humans paratenic (or accidental) hosts, from which it cannot be transmitted back to mosquitoes. *P. knowlesi* infection is viewed as an emerging public health issue because of its severe pathology and the increasing exposure of forest workers to mosquitoes that typically feed on the parasites primary reservoir; long- and pig-tailed macaques [19]. Because passage through this reservoir is thought to be essential for the parasites development, the impact of *P. knowlesi* is restricted geographically to macaque habitats in South-east Asia.

The most important *Plasmodium* species, in terms of their public health impact and economic burden, are *P. vivax* and *P. falciparum*. These have the widest distribution of the five species, and together account for the majority of the diseases' total annual incidence. *P. vivax* causes what was once termed 'benign' malaria, because though causing severe disease the parasite rarely kills [20]. There were an estimated 13.8 million cases of *vivax* infection in 2015, distributed through Central and South America, South Asia, areas of East Africa, and the South Pacific, where the parasite is transmitted by a wide variety of *Anopheles* vectors [13, 21]. *P. falciparum* is responsible for almost all global malaria related mortality. It is transmitted in all sub-tropical regions, but is most abundant in sub-Saharan Africa,

where its dominant vectors are mosquitoes of the *Anopheles gambiae* species complex. The WHO estimates that in 2015 there were 212 million (148-304 million) malaria cases and 429,000 (235,000-639,000) deaths due to malaria [13, 22]. Factors that contribute to these figures include the likelihood of being bitten by an infected vector, the organism's complex pathology in humans, and the outcome of our attempts to control the parasite on both these fronts. On the other hand the number of people at risk of infection, cited as 3.2 billion, is simply a product of the distribution and competence of mosquito vectors [23]. The impact of malaria today therefore reflects the remarkable efficiency and adaptability of *Anopheles* mosquitoes. In his book *The Creation: An Appeal to Save Life on Earth* Professor Emeritus and Pulitzer Prize winning naturalist E. O. Wilson wrote: "It would be a mistake to let even one species out of the millions on earth go extinct". For *A. gambiae*, he reconsidered: "Keep their DNA for future research…and let them go" [24, 25].

Anopheles thrive in harsh conditions. They lay their eggs on the surface of multiple and often temporary pools of water such as collect in pots, animal troughs, and tyre tracks, ensuring genetic variability (by reducing the chance their offspring all hatch from and breed around one site), safety from predation, and rapid population growth even when rainfall is sporadic [26-28]. Breeding site preference, and behaviour during feeding and resting vary between species. The most efficient vectors are specialised to feed on humans (anthropophily), though many prefer to feed on non-human animals (zoophily) or do so only when humans are scarce or vector controls force a behavioural shift in primary host choice [29]. *Anopheles gambiae sensu stricto* is anthropophilic, and enhances its chances of reproduction by biting indoors (endophagy) at times when humans are likely to be asleep, and defenceless. Other vectors show more plasticity in host choice or biting behaviour, making them more robust to the effects of indoor controls like insecticide treated nets and walls [2-4, 29, 30]. Like most blood feeding insects, to facilitate blood-feeding *Anopheles* secrete a mixture of proteins and pharmacologic substances into the skin before and during feeding, many of which are specific to the mosquito's genus and even to the *gambiae* complex [31-33]. Anti-inflammatory and analgesic compounds decrease swelling, redness and pain to maximise the chances of feeding undisturbed [34], while anti-platelet aggregators and vasodilators ensure that blood-flow is unimpeded until the insect has finished feeding.

A vectors efficiency is traditionally defined as its 'vectorial capacity' (C) [35], which is an estimate of the number of mosquitoes that would bite and become infected daily from a single, perfectly infective human. A vectors efficiency may also be considered part of a larger equation describing the efficiency of the parasite it transmits. This 'basic reproduction rate' (R_0) is the number of secondary human infections arising from a single infected human, over the course of a parasites developmental cycle [36, 37]. Vectorial capacity therefore describes the mosquito's raw efficiency, while R_0 describes the likelihood that *Plasmodium* will proliferate in a population. An R_0 of more than one indicates that a disease will remain or spread, as at least 1 secondary infection will result from each primary infection. Less

than 1, and a disease will decline until its elimination. In the recent outbreak in West Africa, the R_0 of Ebola-virus disease was estimated to lie between 1.51 and 2.53 [38]. Estimates for the R_0 of *P. falciparum* in Africa vary from <1 to more than 3000 [39]. This disparity is due in large part to the efficiency of the malaria vector; in Tororo, Uganda, at the turn of the century, individuals were observed being bitten by 160 *A. gambiae* s.s per night [40], providing numerous opportunities for transmission. The real multiplier though is the parasites behaviour within its hosts. The Ebola virus is spread from person to person by contact with infected bodily fluids. Severe symptoms develop rapidly and lead to the host's death within days of becoming infectious, creating a limited window for virus transmission. Left untreated, infective *Plasmodium* may circulate in semi-immune individuals for many months, and even after treatment infective parasites may remain viable for 6-8 weeks [41]. Given their short life span (2-6 weeks) infective mosquitoes are assumed to remain infective for life [42]. A single malaria infected individual may therefore seed hundreds of mosquito infections, thousands if the biting rate is as intense as it was in Tororo, each of which could infect a new person at every feed until the end of its life. Of all the elements of the malaria transmission cycle in the Ross-McDonald equation, reducing the number of mosquitoes biting humans has the greatest impact on the parasites reproductive rate [39]. However, the chronicity of mosquito and human infection is also hugely influential to the parasites ability to survive and reproduce, particularly in populations under intense malaria control.

Pathogenesis and the malaria life cycle

The *Plasmodium* life cycle is complex, passing through motile and immotile, sexual and asexual, and intra- and extracellular forms as it develops in humans and mosquitoes. The parasites genome is haploid for almost the whole cycle, except during stages that emerge in the mosquito after their transmission from capillaries in the skin. The parasites development in mosquitoes is complete once it reverts to haploidy by forming asexual sporozoites, which need to be present in the insect's salivary glands to pass into the human blood supply when the mosquito feeds. Sporozoites develop inside oocysts bound to the mosquito's gut wall, which become implanted shortly after the parasite is ingested (**Figure 1**).

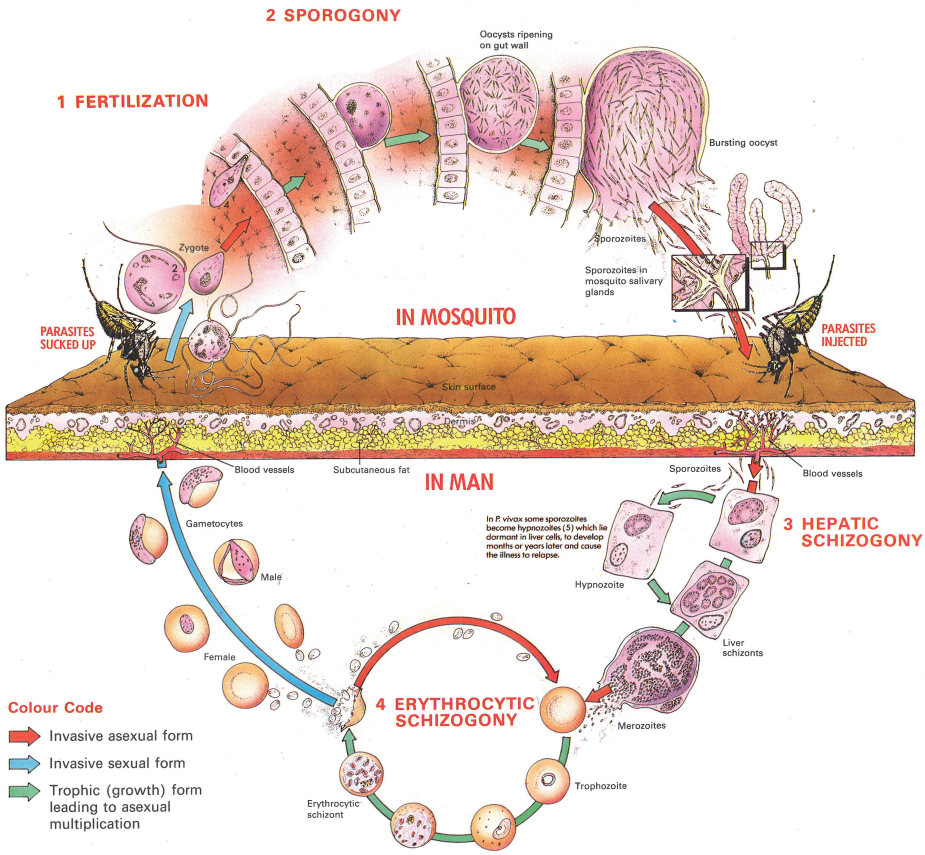

Figure 1. The *Plasmodium* life cycle (A. J. Knell, Malaria: A Publication of the Tropic Programme of the Wellcome Trust, 1991)

When sporozoites are fully formed and have enlarged the oocyst to breaking point (around 10 days after the infective blood-meal), they rupture its walls and are carried in the mosquito's haemolymph to the insect's salivary glands, which they bind to and invade. When an infectious mosquito probes a human's skin in search of a blood-meal, it salivates and may egest sporozoites into the bite wound, most of the time not directly penetrating a vessel but feeding on blood from lacerated capillaries that pools below the epidermis [43, 44]. Sporozoites deposited in the skin migrate through its tissues to enter the blood, where they are quickly carried to the sinusoid vessels supplying the liver [44, 45]. Here they interact with molecules produced by liver cells lining the vessel (hepatocytes and stellate cells) which bind and remove the parasite from circulation [46]. The parasites then enter the sinusoidal layer through Kupfer cells, cross the 'space of disse' underlying the vessels walls, and begin their invasion of the liver parenchyma [47]. After penetrating and migrating

through several hepatocytes, sporozoites are encapsulated in one to form a parasitophorous vacuole. Encapsulated sporozoites then break down and undergo shizogony, developing into thousands of merozoites which are released back into the sinusoids (for *P. falciparum* around seven days after the initial infection) (**Figure 2**).

In the blood, *P. falciparum* merozoites quickly recognise, bind, and invade red-blood cells (RBCs). Within RBCs they form the characteristically ring shaped early trophozoite, and eventually mature into a multinucleated shizont (**Figure 3**). A small number of shizonts become committed to sexual development, forming gametocytes to ensure transmission to the parasites definitive host [48]. The vast majority of shizonts develop asexually, expanding within their human host to increase the transmission potential of future parasite generations [49]. Asexually replicating shizonts each form 16-32 new merozoites, which burst from their first host cell around two days after its invasion to infect new healthy cells.

The life cycle stages preceding the invasion of RBC's by merozoites (collectively, the pre-erythrocytic stages) are non-pathogenic. Malaria symptoms begin after the first waves of cell rupture and invasion that occur during erythrocytic shizogony. Though differing with species and depending on the size of the parasite inoculum and liver biomass, symptoms of *P. falciparum* infection may begin 10 days or so after an infectious mosquitoes bite. Non-severe blood stage *P. falciparum* infection is characterised by a fever that cycles as parasites replicate in the blood, and other symptoms of febrile illness like headache, dizziness, aches, pallor, and nausea. Anaemia begins when the rate of erythrocyte invasion begins to exceed the rate at which new cells are formed, which can continue until so few healthy erythrocytes remain transfusion is required to prevent death. Continued infection compounds the declining cell count both by suppressing the production of new RBCs, and stimulating responses that enhance the clearance of healthy ones [50]. Left untreated, *P. falciparum* can replicate to the extent that more than 10% of all a person's RBCs may be simultaneously parasitized [51]. In real terms, this means that a 50kg child could host more than 19 billion infected RBCs. 'Hyperparasitaemia' of this sort is extreme, but far more scant infections can cause debilitating disease and death, depending on the susceptibility of the host. Symptoms of severe malaria include pulmonary oedema, hyperpyrexia, renal failure, hypoglycaemia, and acidosis [52]. The most deadly effects of *P. falciparum* infection are an artefact of the parasites ability to bind to the walls of small blood vessels in the brain and placenta, a process facilitated by its variant surface antigens (VSA). Though its precise pathology remains unclear, sequestration of parasites in these organs leads to the occlusion of micro-capillaries, leaving parts starved of fresh blood [53]. Cerebral malaria may lead rapidly to coma and death, while placental malaria has often fatal consequences for first time mothers and their foetuses.

Figure 2. Liver cell invasion and asexual development (A. J. Knell, Malaria: A Publication of the Tropic Programme of the Wellcome Trust, 1991)

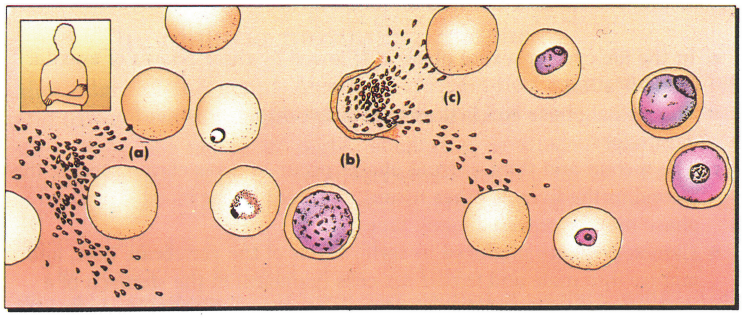

Figure 3. Red blood cell invasion by merozoites (a), asexual development resulting in schizont formation and merozoite formation (b), and merozoite rupture and onward invasion (c) (A. J. Knell, Malaria: A Publication of the Tropic Programme of the Wellcome Trust, 1991)

Because its shizonts produce fewer merozoites, which are limited by their preference for newly formed RBC's (reticulocytes), *P. vivax* infections tend to expand more slowly and to a lower overall density than *P. falciparum* [20]. Febrile illness associated with vivax malaria is severe and quick to take hold, and the parasites formation of dormant hypnozoites in the liver can cause the disease to relapse for years after the inoculating infection [54]. However, because *P. vivax* has a lower multiplication rate (rarely resulting in severe anaemia), and because they cannot sequester in blood vessels, the disease is rarely fatal. In most cases, *P. falciparum* too does not cause severe disease. As demonstrated by the reproductive rate of the Ebola virus, dramatic clinical outcomes are a poor evolutionary strategy. The malaria parasite benefits from a longer period of productivity within and transmission from its secondary host, which is facilitated by the partial immunity conferred by repeated parasite exposure.. Unlike many bacterial and viral diseases though, for which a single exposure can confer protection lasting many years, protective immunity to malaria builds slowly and can be lost rapidly [55]. Severe pathology in falciparum malaria is suffered mostly

by children, who lack immunity, and pregnant women, who are subject to the effects of the parasites placental sequestration [52]. In the first 6-9 months of life infants suffer relatively mild symptoms of disease, but by their first birthday are generally fully vulnerable symptomatic infection. This pattern parallels the decay of maternal antibodies transferred *in utero*, though it is unclear whether these actively protect infants from infection or if they represent a biomarker for maternal exposure that masks other mechanisms of protection [56-58]. Regardless, the first infections following this period of protection are the most dangerous. Surviving children become resistant to severe malaria, but remain vulnerable to febrile illness. By late childhood, continuing or repeat infection builds from partial to clinical immunity, as immune cells become capable of decreasing parasite replication in the blood and lessening symptom severity. Sterile protection against blood stage infection is rarely, if ever observed in nature. Though parasite density and symptom severity may decrease throughout life to the point where infection becomes asymptomatic, there is no evidence of acquired immunity that lessens the likelihood of blood stage infection [59].

The speed with which immunity develops changes from area to area in line with the intensity of local transmission. In areas with intense transmission child mortality may be high, but clinical immunity in surviving children is likely to develop quickly in response to frequent immune exposure. In areas with little transmission or sporadic epidemics, these patterns may be less defined, with clinical immunity developing later in life, and older individuals suffering more severe symptoms because of their low cumulative exposure [60]. In most settings, older children and adults exist in a state of partial immunity, suffering 'uncomplicated' infections and acting as a quiet reservoir of transmission. It is essential here that symptom presentation is not interpreted as an indicator of parasite density or infectivity; though immunity from febrile illness always necessitates some limitation of parasite growth, parasite density in asymptomatic patients may still be high. Asymptomatic infections are also not necessarily benign; infection in the absence of febrile illness may have untold consequences on infant mortality and cognitive development, population productivity, and resistance to other diseases [61]. Transmission intensity and patterns of immunity and clinical presentation have an impact on the way infections and the infectious reservoir are identified.

Immunity and gametocytes

Immune responses to the malaria parasite may be targeted at any of its developmental forms, and valuable findings have been made by observing the response to direct immunisation with various active and inactive forms of the parasite. Exposure to attenuated sporozoites (that do not progress to liver stage infection) and live sporozoites (utilising drugs to supress the blood stages of otherwise viable parasites) induces complete protection in humans and non-human animal models. This 'sterile' protection may act by targeting the exposed sporozoite during its travel to the liver, preventing invasion of the livers cells, or directly

killing infected hepatocytes [62, 63]. In animal models sporozoites inoculated into the skin may be immobilised by sporozoite specific antibodies, including antibodies specific to the thick coat of circumsporozoite protein (CSP) that protects the parasite and facilitates its traversal in both skin and liver [44]. Antibodies specific to sporozoite surface proteins are observed in individuals naturally exposed to the parasites, but their protective efficacy is unknown [64, 65]. Evidence from challenge studies indicates that the sterile protection induced by sporozoites is controlled after hepatocyte invasion by the activation of both CD4+ and CD8+ T-cells [65-68]. However T-cell mediated killing has questionable relevance to the development of natural immunity, having only been observed in response to unnaturally high exposure to sporozoites [68], or sporozoite antigen [69]. In endemic settings adults and children cleared of all infection become re-infected at similar rates, indicating that natural immunity has limited control over the speed and success of pre-erythrocytic development [70, 71].

Perhaps logically, given their relative biomass and longevity, natural immunity to malaria appears to be targeted to the parasites pathological blood stages. Before the end of the Second World War it was common to treat sufferers of neurosyphilis with pyrotherapy, using malaria parasites as a treatment to induce fever and thus clear the bacterial infection. The parasites were incubated in long-term patients who were or became immune to malaria disease after repeated treatment [72]. In 1948, one such patient was exposed to the bites of 2000 *P. vivax* infected mosquitoes, and remained free from blood stage parasites and disease despite developing liver stage infection [73, 74]. The hypothesis that natural immunity was focused on parasites replicating in the blood was confirmed in the 1960's, when adults with clinical immunity were shown to remain asymptomatic after direct inoculation with blood stage parasites [75]. The dominant role of antibody in this protection was shown after passively transferred IgG from immune adults reduced symptoms and parasite density in children suffering severe disease [76]. Anti-malarial antibodies have since been shown to prevent erythrocyte invasion by merozoites [77], activate complement protein fixation [78], stimulate neutrophil respiratory burst [79], opsonise infected cells for phagocytosis [80, 81], reverse rosetting (clumping of infected cells) [80] and prevent cells from binding to the endothelium of the microvasculature [82, 83]. Antibodies specific to numerous parasite proteins have been linked with protection empirically or epidemiologically, including proteins involved in the parasites cell recognition and invasion machinery (Erythrocyte binding antigens; EBA [84], Reticulocyte binding homologue proteins; PfRH5 [84], Apical membrane antigen; AMA-1 & Rhoptry neck protein; RON2 [85], Merozoite surface protein; MSP-1 [78, 86, 87]), and proteins expressed on the erythrocyte surface (encoded by the *var* gene family, including Erythorcyte membrane protein 1; PfEMP-1 & var2csa [80, 82, 83]). However, none of these responses has been linked unambiguously with the development of natural immunity to blood stage infection [88]. Though there is mounting evidence that malaria infection may actively limit the effectiveness of immune memory, the

apparently ineffective development of clinical immunity may also in part be explained by the complexity of protein expression on the surface of infected erythrocytes [55]. PfEMP-1 is trafficked to 'knob' structures that develop on the erythrocyte surface after merozoite invasion, where it facilitates cell binding to ligands in the human microvasculature and thus prevents parasite clearance in the spleen. Individuals exposed to natural infection develop strong antibody responses to the protein, which have been linked with protection from disease [89-92]. However, as it is encoded by a transcriptionally regulated family of 60 *var* genes, each producing a distinct protein variant, the antibody response is only effective while all clonal parasites express a specific protein. Epigenetic mechanisms ensure that only one protein is produced at any time and facilitates spontaneous antigenic variation to ensure that novel variants become fixed in the parasite population in response to immune pressure [93, 94]. By cycling through the *var* gene repertoire the parasite ensures that it can survive for many generations, causing chronic disease and slowing the development of clinical immunity [95-97].

The final stage exposed to the immune system within humans is the infective gametocyte. As the parasites entire reproductive potential depends on this small portion of its total biomass, it is essential that gametocytes are able to avoid recognition and clearance. As for the VAR gene family, sexual commitment in shizonts is controlled epigenetically [93]. Sexual development is initiated by a signalling pathway during shizont development that releases the Apatella2-g (*ap2-g*) gene from transcriptional regulation, causing all the cells daughter merozoites to express the protein. When these invade new erythrocytes, instead of proceeding through continued rounds of asexual endomitotic replication, each produces a haploid gametocyte [98, 99]. Each sexually committed shizont produces daughter cells of a single sex, and in natural infections the resulting gametocytes exist at a ratio of approximately 3-5 females to 1 male [100, 101]. *P. falciparum* gametocytes take 10-12 days to mature to a stage at which they are ready to infect mosquitoes. They do this in five stages, the first two of which are morphologically indistinguishable from immature trophozoites. From stage III onwards the cell elongates to become the characteristically crescent shaped mature gametocyte (**Figure 4**).

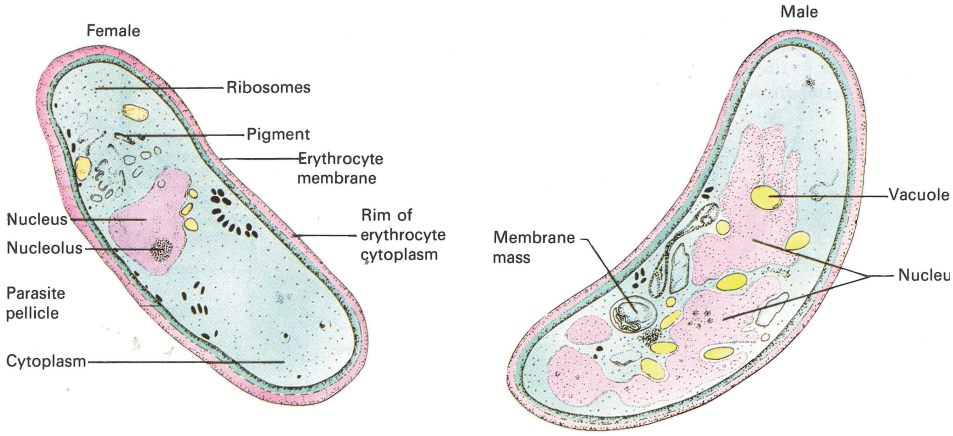

Figure 4. Female and male mature (Stage V) *P. falciparum* gametocytes (A. J. Knell, Malaria: A Publication of the Tropic Programme of the Wellcome Trust, 1991)

Unlike asexual parasites, gametocytes cannot bind to blood vessel walls because they appear to lack parasite-derived erythrocyte surface proteins. This is a double edged sword; the gametocyte remains immunologically silent, shielded within a relatively unchanged erythrocyte, but because they cannot sequester by binding to the microvasculature they risk splenic clearance. Instead, immature gametocytes sequester in the extravascular compartments of the bone marrow and spleen [102-104]. Uninfected erythrocytes and immature gametocytes can pass into and out of these areas, but gametocytes between stages II-IV appear to reduce the deformability of their host cells to such a degree that they cannot escape to re-enter the circulation [105, 106]. Sequestration may therefore either be active, involving enhanced production of gametocytes in these extravascular spaces, or passive; they may simply be retained [105]. There is also very recent evidence for the presence of previously unidentified ligands on the early gametocyte surface, which may facilitate homing or binding to erythroblastic islands in the bone marrow parenchyma (Dantzler, *in press*). What is clear is that when gametocytes approach maturity, they once again become flexible, egressing the bone marrow into the blood, where they are once again vulnerable to immune recognition and clearance. Though there is a hypothesis that antibody responses to unknown proteins on the surface of intact gametocytes may limit gametocyte density, the lack of known parasite-derived surface antigens makes it unlikely that antibodies acting directly on gametocytes would have significant effect on their infectivity [107]. However, this is not to say that humans do not produce important antibody responses to gametocytes. Mature gametocytes express a raft of unique proteins, as well as many RNA transcripts that are then subject to translational repression until the parasites development in mosquitoes [108, 109]. Though many proteins essential to parasites development in the mosquito are part of the 'repressome', others are fully expressed by the gametocyte within humans,

so elicit antibodies when they break down and become exposed to immune cells in the blood. Antibodies produced against these proteins form the basis of naturally acquired 'transmission reducing immunity' (TRI), wherein antibodies from individuals exposed to malaria infection actively inhibit parasite development in mosquitoes [110, 111].

Plasmodium transmission hinges on the successful development of the parasites inside mosquitoes. Inside the mosquito gut, gametocytes become activated by mosquito compounds, and changes in temperature and pH, to undergo gametogenesis. Female gametocytes become spherical, and secrete proteins that enable them to emerge from their erythrocyte host cell [112, 113]. Male gametogenesis is more active. Male gametocytes undergo 3 rounds of mitotic division to form 8 motile microgametes, which emerge from the erythrocyte in a process called exflagellation (**Figure 5**).This is why natural gametocyte sex ratios are female biased. The ideal strategy would be for the parasite to produce 8 females for every male; enough to result in a gamete ratio of 1:1 [101, 114, 115]. Because gametocyte ratios vary based on the degree of fertility competition with co-infecting strains [100] or red blood cell and gametocyte density [49], a lower female bias is generally observed, increasing the likelihood of fertilisation at the expense of its theoretical potential [49, 116].

To enhance their chance of locating a female macrogamete in the pre-digested blood meal, exflagellating male gametes bind neighbouring blood cells to form exflagellation centres. Proteins on the gamete surface facilitate the recognition and binding of male gametes to the female, while secreted proteins enable their invasion and fertilisation [117]. Translationally repressed female gametocyte proteins are activated at this stage, enabling the resulting diploid zygote to form a motile ookinete in the gut lumen. The ookinete breaks through the peritrophic matrix that normally retains the particulate content of a blood meal, and traverses the inner layers of the gut epithelium to become embedded in its basal layer [42] (**Figure 6**). Here the parasite arrests, forming an oocyst which develops slowly and harbours the sporozoites that make the mosquito infective. In nature, mosquitoes usually harbour less than five oocysts, each of which may contain several thousand sporozoites [118].

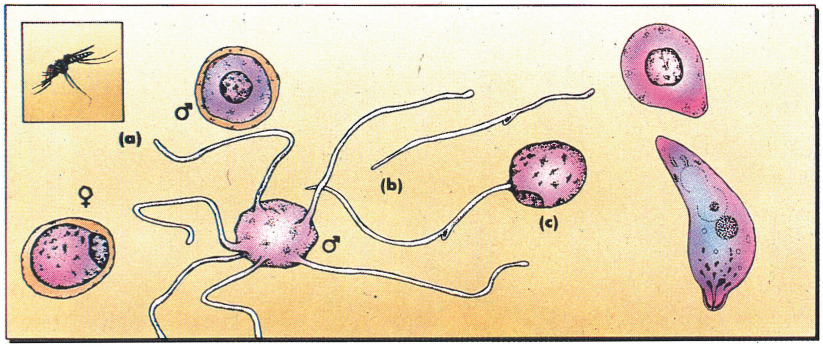

Figure 5. Gametocyte activation in the mosquito midgut (a), gamete formation (b), fertilisation of macrogametes (female) by microgametes (male) (c), and subsequent life stages (right: zygote/ retort [top] and ookinete [bottom]) (A. J. Knell, Malaria: A Publication of the Tropic Programme of the Wellcome Trust, 1991)

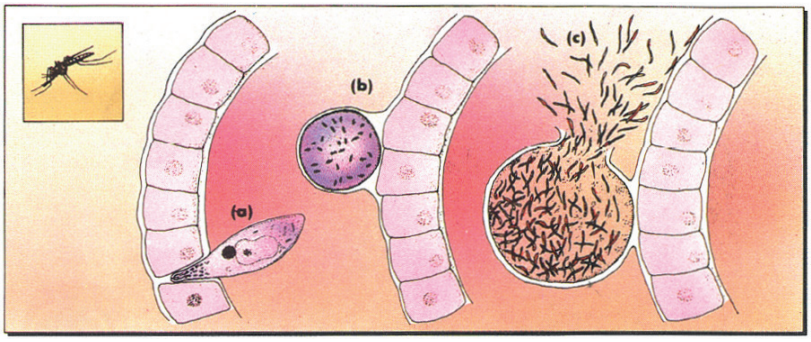

Figure 6. Ookinete invasion of the mosquito midgut epithelium (a), oocyst formation and sporozoite budding (b), and oocyst rupture and sporozoite release into the mosquito haemolymph (A. J. Knell, Malaria: A Publication of the Tropic Programme of the Wellcome Trust, 1991)

The transition between life stages leading to oocyst formation occurs rapidly, with ookinete implantation occurring after 16-32 hours of the blood meal in *P. falciparum*. Despite this speed, estimates of the total population loss between the gamete and oocyst stages are as high as 99.4% [118]. This loss appears greatest before or during gamete fertilisation, though ookinetes may also fail to penetrate the midgut or be destroyed in the process, and successfully embedded ookinetes may also arrest in the midgut epithelium, and fail to develop into productive oocysts [118-121]. The vulnerability of these exposed life stages to chance, to human and mosquito factors in the mosquito gut, and to their low and precipitously decreasing density make mosquito stage parasites an ideal target for malaria control; one in which relatively small disturbances may result in total reproductive failure.

Elimination

The aim of malaria control is to reduce mortality and morbidity caused by the disease. Our success in doing this since the turn of the century is only apparent because it contrasts so starkly with the period before. The early 1990s saw an unprecedented public health crisis in Africa, with malaria outbreaks of 'epidemic proportions' causing 300-500 million cases and 1.7-2.5 million deaths annually [122, 123]. Though political anergy and poor health infrastructure bear some responsibility, the crisis was catalysed by antimalarial drug resistance. Chloroquine had been the first-line antimalarial since the early 1950's, and though resistance to the drug was observed as early as 1957 it remained effective in Africa until the late 1970's [124, 125]. Sulphadoxine-pyrimethamine, a drug retracted decades previously due to the widespread emergence of resistance, was reintroduced as an alternative in the 1990's only to suffer the same fate within years [126, 127]. Hailed as the key to 'averting a malaria disaster', artemisinin derivatives became widely used for the treatment of uncomplicated malaria in the early 2000s following mounting evidence of their tolerability and efficacy *in vivo* [128]; one key study showed they cleared 10-100 times more blood stage parasites over a single asexual cycle than other antimalarials [129]. To avoid the fate of previous treatments, the WHO advised against all artemisinin monotherapies in 2007, recommending that they be combined with slower acting partner drugs to avoid parasite recrudescence. In Ghana, Artemisinin combination therapies (ACTs) gave >97% clearance by four weeks after treatment while Sulphadoxine-pyrimethamine and chloroquine resulted in 60% and 25% clearance respectively [130]. ACTs have remained 'first line' in all malaria endemic areas, and their effectiveness is unchanged in most of Africa. In South-east Asia though, widespread increases in parasite clearance time after treatment has marked the emergence of artemisinin resistance [131]. Resistance is now known to be caused by mutations of the parasite gene *kelch13*, and resistant strains associated with these mutations have spread throughout the mainland from Vietnam to northern Myanmar [132, 133]. Thankfully the same mutations have yet to be observed in Africa, though increased clearance times have been reported in Kenya [134, 135]. As alternative drugs are still some distance from licencing and production at scale, resistance in Asia poses a major threat to malaria control globally. If the spread of these parasites continues unchecked, their eventual observation in Africa is inevitable. Containment of resistant parasite strains is therefore of the upmost importance if the recent successes of malaria control are to be sustained, and local elimination of (resistant) falciparum malaria may be the only viable solution. At the same time, declining cases and deaths have galvanised the public health community into a global call for the parasites eradication [136].

Elimination of any infectious disease is achieved when the number of cases acquired in a defined area is reduced to zero. More formally, elimination status is granted after consolidation of this transmission level for three years [137, 138]. Eradication is achieved when the disease agent is eliminated globally. The only infectious diseases to have been

eradicated by human efforts are the viral diseases smallpox and rinderpest, both of which were eradicated with highly effective vaccines and rigorous surveillance [139, 140]. The only parasitic disease determined to be fully 'eradicable' is Dracunculiasis, for which individual cases are easily diagnosed, treated, and prevented from resulting in further transmission [141]. Malaria eradication is a far more complex prospect, particularly because it has failed before. In 1955 a global malaria eradication programme (GMEP) was initiated, imposing a rigid system of indoor spraying with DDT, and treatment of symptomatic cases with chloroquine [142]. By the late 1960's, the WHO acknowledged the GMEP's failure, and recommended a return to control rather than eradication. Though it suffered controversial social and operational problems, the GMEP's inability to sustainably halt transmission has been blamed on its initial unwillingness to acknowledge that malaria transmission intensity varies with epidemiological and geographical context [6, 143]. In light of the first GMEP's failure, the prospect of a renewed program of malaria eradication has been regarded with understandable caution. Five countries have achieved elimination in recent years, all of which are in the Eastern Mediterranean region and suffered low endemic transmission before pushing to eliminate [136]. Of the 21 countries identified as being capable of elimination by 2020, six are in the African region, and again all have stable low transmission. The heterogeneous nature of malaria transmission, which is present at all levels of endemicity but becomes more apparent as transmission is reduced, is now recognized as a major barrier to effective transmission reduction [144-147]. Another will be that the worst affected areas are some of the least wealthy and politically stable; 35% of all deaths due to malaria occur in Nigeria and the Democratic Republic of Congo [136]. In areas with moderate and high transmission intensity (most of sub-Saharan Africa), traditional controls are unlikely prevent local transmission. Griffin *et al.* estimated that with 80% coverage with long lasting insecticide treated nets (LLINs), treatment with ACTs, mass screening and treatment programs (MSAT), and an efficacious pre-erythrocytic malaria vaccine, areas with high level transmission would need additional tools or major social reform to bring parasite prevalence below 1% [148]. The call for malaria eradication has therefore been met not with a pre-defined program to achieve it, but with an agenda to focus research and development toward making elimination possible where it currently is not, so that eradication is put within reach. Much of this hinges on shifting the malaria control paradigm from case prevention to transmission reduction. Though reduction of transmission is the natural result of intensive control, it may be hastened by ensuring coverage of those individuals who are most infectious to mosquitoes, or using tools specifically designed to reduce human-mosquito transmission.

Measuring infectiousness

During the GMEP, Robert Muirhead-Thomson, an entomologist at the Liverpool school of tropical medicine, observed that following extensive reductions in malaria transmission "the question of bridging the gap between malaria control and malaria eradication may well depend on a more accurate appraisal of the remaining source of infection in the human population" [149]. In community surveys, around two thirds of infections detected by microscopy are asymptomatic [150, 151], so would go untreated if case detection was passive. Even if surveillance were active, molecular diagnostics have revealed that a large proportion of infections in endemic populations circulate at densities below the detection threshold of microscopy or rapid diagnostic tests (~40 parasites per μl in rigorous assessments, but closer to 100/μl during standard surveillance). In low endemic settings, sub-microscopic infections may account for as much as 88% of the infected population [152]. As gametocytes represent only 1-2% of the total parasite biomass, the proportion of the population that is hypothetically infectious is even more heavily underrepresented in standard surveillance [153]; an estimated 91.4% of gametocyte infections are missed by microscopy [152]. Worryingly, mathematical models show that where controls are focused on standard case detection and control, a sub-microscopic parasite reservoir is sufficient to maintain transmission [154]. Because of the frequency of gametocyte infections, it makes sense that an 'any is too many' strategy is adopted, wherein parasite infection, symptomatic, gametocytaemic, or otherwise, is treated as potentially infectious [151, 155]. However, not all gametocytaemic individuals are likely to contribute equally to onward transmission.

At the individual level, the density of gametocytes in the blood that are detectable by microscopy is a poor predictor of infectiousness to mosquitoes [156]. Gametocytes can be quantified with highly sensitive messenger RNA based assays, but so far these have not crystallised the relationship between their density and infectivity. Though high densities (>200 gametocytes per μl of blood) are more likely to infect [157], various intrinsic parasite and human factors affect the chance of establishment in the mosquito, with the result that many sub-microscopic gametocyte infections prove infectious, and some high density infections appear sterile [158-160]. Molecular gametocyte detection is currently based on the detection of *Pfs25* mRNA [161, 162]. Though abundant and highly specific to gametocytes, recent transcriptomic analyses show that *Pfs25* is transcribed primarily by female gametocytes [163-165], so while useful for determining gametocyte presence, the most advanced gametocyte detection tools so far available are now known to provide inaccurate estimates of total gametocyte density.

Several molecular markers for asexual and sexual stage parasites are in development, and have been used in concert to estimate the stage-specific composition of natural infections [166]. Markers specific to male and female gametocytes [165], and to different stages of their development [106, 167] will bring us closer to developing RNA based assays capable of accurately quantifying gametocytes and predicting their infectivity. Gametocytes only

recently released from sequestration are less infective than gametocytes that have had the time in circulation to produce sufficient quantities of proteins and mRNAs that facilitate fertilization and establishment in the mosquito [168, 169]. PHISTa and PfGEXP5 have been identified as markers of early gametocytes [166, 170], while PF14-0367 and Pfs25 are enriched in mid-stage and mature gametocytes respectively [166]. Separate male and female specific markers would enable to total gametocyte density to be quantified accurately, and provide estimates of gametocyte sex ratio, which is critical to the likelihood of successful fertilisation in the mosquito, and may be affected by total gametocyte density, by the duration and multiplicity of infection, and by drugs with different levels of activity against male and female gametocytes [116, 171, 172]. Sex specific gametocyte quantification is so far limited in sensitivity [165]. Different parasite strains may also be differently infectious, either producing more gametocytes or varying in their vulnerability to drugs or immune factors that might inhibit transmission [173].

Human factors are also capable of effectively sterilising viable gametocyte infections. Because gametocytes are hidden within the erythrocyte while circulating in the blood, human antibody responses to gametocyte proteins are more likely to affect the success of parasite development in mosquitoes than the number of gametocytes in circulation [174]. Though there is evidence that naturally acquired TRI is short lived [175-177], develops in response to recent, high gametocyte exposure [110, 177], and is correlated with the presence of antibodies specific to well characterised proteins involved in gamete fertilisation (Pfs230 and Pfs48/45) [110, 111], our understanding of the dynamics and causal mechanisms of TRI is poor. Transmission reducing immunity has been investigated by performing ELISA to detect antibodies specific to Pfs48/45 and Pfs230, but because these responses do not fully explain the occurrence of TRI [156, 178], serological assessments cannot yet predict the effect of immune factors on gametocyte infectiousness.

Because its effectors are so numerous, the only way of comprehensively measuring infectivity is to feed mosquitoes on a potentially infectious individual's blood and observe whether the insects become infected (in direct skin or membrane feeding assays; DSFA/DMFA) [179, 180]. Xenodiagnoses of this sort are extremely labour intensive, so can only realistically be considered an epidemiological tool, rather than one than might be used in standard surveillance. The presence of TRI may be determined in the direct membrane feeding assay (DMFA) by reconstituting a potentially infectious person's blood cells in serum of someone with no exposure to malaria [181], but this only adds to the complexity of standard feeding assays. By purifying all antibodies from a blood sample, their effect on gametocytes grown in culture may also be tested in the standard membrane feeding assay (SMFA) [111, 182]. Like the DMFA this assay is restrained by the skill and work load required to assess mosquito infection status, by manual dissection. So far these are the gold standard assays for predicting or measuring the infectiousness of natural or cultured gametocytes.

Mosquito feeding assays are rigorous and inclusive as a measure of individual level infectivity. However, the infectious reservoir (the collective body of individuals in a population capable of infecting mosquitoes) is shaped by extrinsic factors that affect the likelihood of infectious individuals actually infecting mosquitoes. Mosquitoes respond to infection by mounting an innate immune response, which varies in effectiveness in different mosquito-parasite species combinations [183], even between different members of the *A. gambiae* subfamily [173, 184]. Human factors too may affect the shape of the infectious reservoir, as individuals are likely to be differently exposed and attractive to mosquitoes, depending on their age and size [185], their protection from biting [186], their proximity to breeding sites and permissive mosquito species [187], and a range of other factors. Understanding infectivity at a population level requires that extra-individual barriers to gametocyte transmission potential be considered.

Transmission reduction

Where their efficacy and population coverage are high, insecticide treated nets and effective schizonticidal drugs are capable of effecting significant reductions in transmission [148, 188, 189]. Improving our understanding of who fuels transmission to mosquitoes may help facilitate this reduction, but in most areas with historically high intensity *P. falciparum* transmission, intensively applied conventional controls have proved unable to completely prevent transmission [190-192]. The diminishing returns of increased net provision in areas of high coverage [193], and the burgeoning importance of outdoor biting mosquito species in maintaining residual malaria transmission [194] highlight the need for novel tools which directly combat human infectivity.

Because of their ability to immediately reduce human infectivity [195], and because they may be used to prevent the spread of drug resistance [196], there is great interest in drugs that target mature gametocytes specifically. Artemisinins are active against stage I-III gametocytes, so they more rapidly decrease transmission potential than older more exclusively schizonticidal antimalarials [197-200]. Mature gametocytes appear robust to the effects of standard artemisinin doses *in vivo,* so though they reduce infectivity compared to non-artemisinins, they do not completely prevent transmission [41]. Only the 8-aminoquinoline Primaquine, which has been used since the 1950's for preventing *P. vivax* and *P. ovale* relapse due to its activity against liver hypnozoites [201], is currently licenced as a gametocytocide. At therapeutic doses primaquine is only active against the asexual liver and sexual blood stages of *P. falciparum* [202, 203], so it has either been used as a prophylactic [204] or combined with a shizonticide as a single dose treatment for the reduction of gametocyte transmission [205]. Opposition to the drugs more widespread use is largely based on its toxicity in individuals with glucose 6-dehydrogenase (G6PD) deficiency, a common x-linked trait that results in heightened risk of acute haemolytic anaemia after treatment [206]. The severity of primaquine toxicity in G6PD deficient

individuals is dependent on dosing; millions of patients have received primaquine therapy for *P. vivax* and only 13 G6PD related deaths have occurred, all having received repeated high doses. Though incidences of acute haemolysis have occurred in G6PD deficient males after a doses of ~0.5 base/kg primaquine, no deaths have been associated with any single dose regimen [206]. As primaquine is rapidly metabolised and the half-life of its metabolites is very short, a single dose of 0.75 mg base/kg was regarded as safe in G6PD deficient individuals for the prevention of *P. falciparum* transmission until 2010, when use of a lower dose of 0.25mg/kg was advised for use in areas aiming to eliminate, or areas at risk from artemisinin resistance [205, 206].

The search for new gametocytocides is high on the research agenda for malaria elimination, and though there is some hope [207], the complexity of screening drugs with outcomes manifest as reduced mosquito infection is a major stumbling block for the development of transmission reducing drugs [208]. *In vitro* alternatives exist for assessing the viability of cultured parasites after exposure to potential transmission reducing agents, but these only interrogate effects active on the gametocyte, which either kill or prevent them from activating to form gametes [209-212]. The same is true for vaccines designed to interrupt malaria transmission (VIMT). As with drugs, any vaccine that reduces liver or blood stage parasite reproduction may inhibit transmission to mosquitoes by limiting gametocyte production. Investment has so far largely focused on pre-erythrocytic and blood-stage vaccines, which afford both personal protection against disease and, if potent enough, reduced infectivity. Though falling short of the minimum requirements for protective efficacy and duration proposed by the WHO [213], the licencing of the RTS,S vaccine in 2015 was a significant milestone in infectious disease control [214]. This said, reducing infectivity in enough individuals to cause community wide transmission reduction requires more stringent thresholds for vaccine efficacy. Sub-optimal pre-erythrocytic or blood stage vaccines may be complemented by VIMT [215] eliciting antibodies capable of neutralising parasite [215, 216] or mosquito [217, 218] proteins involved in the parasites sexual developmental pathway.

Antimalarial drugs can curb the long infectious period in infected individuals by abrogating asexual stage multiplication and preventing gametocyte production (all effective schizonticides) and specifically clearing mature gametocytes. However, the public health impact of these measures will be largely determined by the proportion of the total parasite reservoir that is successfully targeted, and the frequency and timing of their application. Gametocytocidal drugs are generally discussed in the context of mass administrations, which may have an important role in regional elimination [195]. Models suggest that the addition of specifically gametocytocidal drugs may be less important during MDA than ensuring maximal coverage with effective schizonticides [219]. In areas where the effectiveness of current treatments is waning, gametocytocides are likely to have significant utility [196]. In the broader context of transmission reduction though, a VIMT is highly desirable. A single

round of vaccination with a VIMT that reduces infectiousness to zero for the length of a transmission season, plus the time it takes for the human infectious reservoir to be cleared naturally, could effectively wipe-out the mosquito infectious reservoir and eliminate malaria. Coverage, immunogenicity, and longevity are the barriers to this 'ideal public good' [220], but even a partially effective vaccine overcomes many of the disadvantages of transmission blocking drugs, like sub-optimal treatment adherence, short half-life, long-term expense, and genetic effects on metabolism and safety [206, 221].

* * *

The chapters of this thesis, **Malaria: what affects who infects?** are concerned with the measurement of gametocyte infectivity, and with the evaluation of interventions or intrinsic factors that affect individual infectivity and contribution to the population infectious reservoir. Understanding how the infectious reservoir changes as transmission decreases may enable tools to be targeted more effectively, by focusing on areas or populations in which transmission may be maintained and seeded from after intervention. Understanding and measuring individual level infectivity is essential for the development of transmission reducing interventions, and their evaluation in endemic settings.

Quantifying heterogeneity in parasite and mosquito exposure

Malaria transmission and clinical burden are highly heterogeneous at all levels of endemicity [144-146]. One approach to improving the effectiveness of interventions is to target them to individuals who are most regularly bitten and infected. Within a focus of malaria transmission (an area around a mosquito breeding site where transmission is maintained throughout the year), some areas host greater than average transmission, resulting in high local infection rates and clinical incidence [144, 145]. These 'hotspots' may form because of poor housing quality and proximity to breeding sites, among other social and geographical factors [222]. Their identification enables control targeting to maximize the impact of interventions, but is also important because these may be the hardest populations in which to achieve and sustain elimination [39]. Hotspots may be detected by determining the spatial distribution of current malaria infection and of prior exposure at a population level, which represent biomarkers for malaria risk and potential infectivity [145]. Current infection is best determined using sensitive molecular diagnostics, while previous infection (and possibly current risk) can be interpreted from serologic assessments of malaria-specific antibody titre. The distribution of antibodies among individuals of different age within populations provides a valuable indication of change in transmission intensity over time [223]. At another level, exposure to mosquitoes may be measured to determine whether individuals in areas with a high risk of infection are also likely to be bitten and contribute to the mosquito infectious reservoir. Previous studies indicate vector exposure may be determined serologically, by determining the presence of antibodies to vector saliva [224-228].

Understanding the human infectious reservoir for malaria

Whilst reliable measures to assess spatial variation in malaria infection, infection risk, and vector exposure support targeted interventions, the impact of hotspot-targeting is not self-evident. A recent clinical trial examining the efficacy of interventions targeted to serologically defined hot-spots of malaria exposure concluded that the effects of intensified vector and treatment based controls were only effective within intervention areas, and then only transiently, and did not influence transmission to a wider geographical area [229]. The trial illustrates our limited understanding of the units of malaria transmission in endemic settings, our failure to grasp how incident infections in naturally exposed humans and mosquitoes are related spatially and temporally, and what the unit of targeting should be to effectively interrupt local transmission. Part of the uncertainty lies in the fact that the distribution of infectiousness does not necessarily correlate with the distribution of disease incidence or vector exposure. Improving our understanding of the human infectious reservoir could make plain the failings of current control deployment strategies for interrupting malaria transmission. It may be that effective transmission reduction requires more direct targeting of the human infectious reservoir. Gametocyte prevalence and density are highest in infants and young children, decreasing with age as cumulative exposure and acquired immunity to the parasite builds [74, 230, 231]. Whilst this suggests that children may be the primary drivers of transmission, the relationship between gametocyte densities and *de facto* contribution to transmission is obscured because children are generally well protected by nets during the evening, when mosquitoes are seeking blood meals, and may also be less attractive than adults. Though less objectively infective, adolescents and adults make up a larger proportion of the population, may be more bitten more frequently, and so may contribute more to the infectious reservoir. These variations in immunity and mosquito exposure vary even between members of the same house. It is important they are understood even if transmission focused interventions are not targeted.

Tools to assess infectivity and develop transmission-blocking interventions

From a public health perspective, the ultimate reason to understand the infectious reservoir is to target it and prevent onward transmission. Because conventional controls do not address the growing problems of outdoor mosquito biting and residual malaria transmission [232], transmission-blocking drugs and vaccines are considered important components of efforts to expedite elimination [233, 234]. Both the laboratory based development of drugs and vaccines and the trial evaluation of these interventions in the field require assays to measure transmission from humans to mosquitoes. In the lab SMFA can be employed to test the inhibitory effects of drugs, or naturally acquired antibodies such as might be collected from pre- and post-vaccination sera during vaccine trials, on the infectiousness of cultured gametocytes [208, 235]. Field based DMFA are more restricted to testing the infectivity of

naturally acquired gametocytes [181]. Both assays are expensive and logistically difficult, primarily because the readout, the quantity of successfully established oocysts in the mosquito gut, normally requires pain staking dissection and microscopic examination of large numbers of mosquitoes [236]. Where cultured gametocytes are used enhancements in scalability are made possible by changing the method of quantifying mosquito infection intensity. Detecting a marker of mosquito infection rather than counting oocysts could increase the assays scalability, and would have the additional benefit of making its readout entirely objective.

Primaquine is the only currently available drug that actively sterilises *P. falciparum* infection, and despite an increasing number of reports of its safety and efficacy at single low dose in G6PD deficient individuals, concerns remain over its use in mass drug administrations (MDA) [206]. There is therefore a need to identify novel transmission blocking drugs. These should have a safety profile that permits their use in MDA, and ideally broad spectrum activity against asexual parasites and their gametocytes (for use in areas of ACT resistance). Such assessments would benefit from increases to the SMFAs throughput and objectivity.

In the field, using transgenic parasites in feeding assays is impossible, but increases in throughput are still required for assessments during population level trials of transmission reducing drugs and vaccines. If mosquitoes with single oocysts are as capable as causing human infection as mosquitoes with multiple oocysts, then the quantity of infection is less relevant and feeding assays made operationally attractive by replacing dissection with quicker methods of detecting rather than quantifying mosquito infection.

Understanding and preventing malaria transmission in endemic areas

The DMFA is the gold standard measure of human infectiousness, because its output (mosquito infection) is subject to all of the intrinsic determinants of gametocyte infectivity. Since the mid-20th century it has been established that some individuals do not infect mosquitoes, despite carrying many gametocytes [149, 160, 237]. In addition to their absolute quantity, and compounds or enzymes in the blood that may affect parasite survival, two key factors affect the likelihood of gametocytes in human blood successfully infecting mosquitoes; gametocyte sex ratio, and transmission reducing immunity (TRI). The relationship between gametocyte density and infectivity generally appears non-linear, but previous assessments have either been based on estimates made by microscopy, or on molecular assessments which used sub-optimal female specific assays [157]. Accurate quantification of male and female gametocytes separately would allow reliable assessment of the relationship between total gametocyte density and gametocyte sex-ratio with infectivity. Such an assay would also allow investigation of factors with putatively sex-specific effects, such as the gametocytocidal drug primaquine [196].

Naturally acquired transmission-reducing immunity

Though we have known of the existence of TRI for decades, and even induced it by inoculation with sexual stage parasites [233, 234] and recombinant gametocyte proteins [238-240], its mechanisms have remained unclear. Because we are unaware what antibody responses to malaria antigens are responsible for the phenomenon, investigation of the impact of TRI on natural malaria transmission has been very limited. At present, studies are restricted to determining the statistical associations of infectivity and responses to gametocyte proteins known to induce TRI after immunisation (Pfs48/45 and Pfs230). The development of the few transmission-blocking vaccines currently in development proceeded from the identification of sexual stage antigens with essential roles in gamete fertilisation [235, 236], but antibody responses to other sexual stage proteins expressed by gametocytes are likely to contribute to transmission inhibition [178, 237].

A better understanding of the phenomenon of naturally acquired TRI would provide insight into an essential component of human infectivity, and possibly, new starting points for TBV development.

Objectives

For the development of transmission reducing drugs and vaccines, and translating their success from the laboratory to the field, measures of *Plasmodium*'s infectivity to mosquitoes are essential. Malaria elimination, particularly local elimination in areas aiming to contain the spread of artemisinin resistant parasite strains, requires rigorous surveillance of the infectious reservoir and efficacious and operationally attractive approaches to clear this reservoir. The aim of this thesis is to investigate the effectors of human malaria infectiousness to inform the development and application of transmission-reducing interventions.

In **Chapter 2**: **Combined DNA extraction and antibody elution from filter papers for the assessment of malaria transmission intensity in epidemiological studies,** we aim to develop and validate a tool for simultaneously assessing malaria prevalence and prior exposure during epidemiological studies, and population surveillance. This could have utility for intervention targeting, either to individuals with current infection, or to individuals within foci of high malaria risk. In **Chapter 3: IgG responses to *Anopheles gambiae* salivary antigen gSG6 detect variation in exposure to malaria vectors and disease risk**, we aim to validate a serological assay detecting antibody responses to a recombinant *Anopheles* specific salivary protein, gSG6. We hope to show the assays utility for the measurement of exposure to malaria vectors, and determine whether exposure to malaria vectors is associated with historical malaria incidence. This assay may have use in combination with population assessments of malaria infectivity. In **Chapter 4: Assessing the infectious reservoir of *P. falciparum*: Past and future**, we review the available literature on the infectious reservoir, and highlight the importance of direct assessments of infectivity and their inherent weaknesses. We aim to stimulate discussion on the future role of mosquito

feeding assays in malaria surveillance, and to suggest methods for the improvement of these techniques as measures of the infectious reservoir.

In **Chapter 5: A scalable assessment of *Plasmodium falciparum* transmission in the standard membrane-feeding assay, using transgenic parasites expressing Green fluorescent protein–Luciferase,** and **Chapter 6: A semi-automated luminescence based standard membrane-feeding assay identifies novel small molecules that inhibit transmission of malaria parasites by mosquitoes**, we aim to develop a novel iteration of the standard membrane feeding assay (SMFA), for the laboratory assessment of *Plasmodium* infectivity. Specifically, we aim to increase the throughput and objectivity of the assay by using transgenic, luminescent parasite cultures, which should eliminate the need for mosquito dissection. Such assays may have utility in the development of transmission-reducing interventions prior to their application in the field. They may also have more direct benefits to the assessment of infectivity of endemic populations, by providing rapid assessments of transmission-reducing immunity. In the field, increases in the throughput of assessments of infectivity can be achieved by similarly removing the need to dissect mosquitoes to assess their infection rate. In **Chapter 7: The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays**, and **Chapter 8: A comparison of *Plasmodium falciparum* circumsporozoite protein-based slot blot and ELISA immuno-assays for oocyst detection in mosquito homogenates**, we aim to provide evidence that mosquito infection prevalence (determined at time points amenable to safe and rapid assessment) predicts eventual mosquito infectiousness. In so doing, we hope to open the way for rapid screening with mosquito feeding assays, which could ease the assays well known operational difficulties. Such improvements may support bringing successful transmission-reducing interventions to fruition in the field.

At the next level, if new molecular and serological readouts can be shown to provide acceptable proxies for infectiousness, field based assessments of malaria infectivity may be set aside altogether. Though such tools may be a long way from realisation, we aim to investigate key effectors of malaria infectiousness; gametocyte density, gametocyte sex, and transmission-reducing immunity. In **Chapter 9: Predicting mosquito infection from gametocyte density and sex-ratio**, and **Chapter 10: Gametocyte sex ratio and infectivity after dihydroartemisinin-piperaquine with primaquine: Results from two randomised, controlled trials**, we aim to develop a new, sensitive, molecular assay of gametocyte density, capable of predicting the densities of male and female gametocytes separately. We aim to utilise this assay to re-assess findings previously published on the relationship between total gametocyte density and infectivity with a new focus on sex-ratio and as the readout of clinical trials with primaquine, a gametocytocidal drug thought to preferentially clear male gametocytes. Finally, with **Chapter 11: Naturally acquired immunity to sexual stage *P. falciparum* parasites**, and **Chapter 12: Unravelling the immune signature of *P. falciparum* transmission blocking immunity**, we aim to discuss and investigate the impact

of transmission-reducing immunity on malaria infectivity. We aim to clarify the role of naturally acquired antibodies to proteins known to induce TRI after immunisation as recombinant proteins, and to uncover additional biomarkers of TRI if these exist. We aim to study the association of antibody responses to these known and unknown proteins with infectivity in field assessments of malaria infectivity, bridging the gap between lab and field based assessments of the impact of TRI on malaria transmission.

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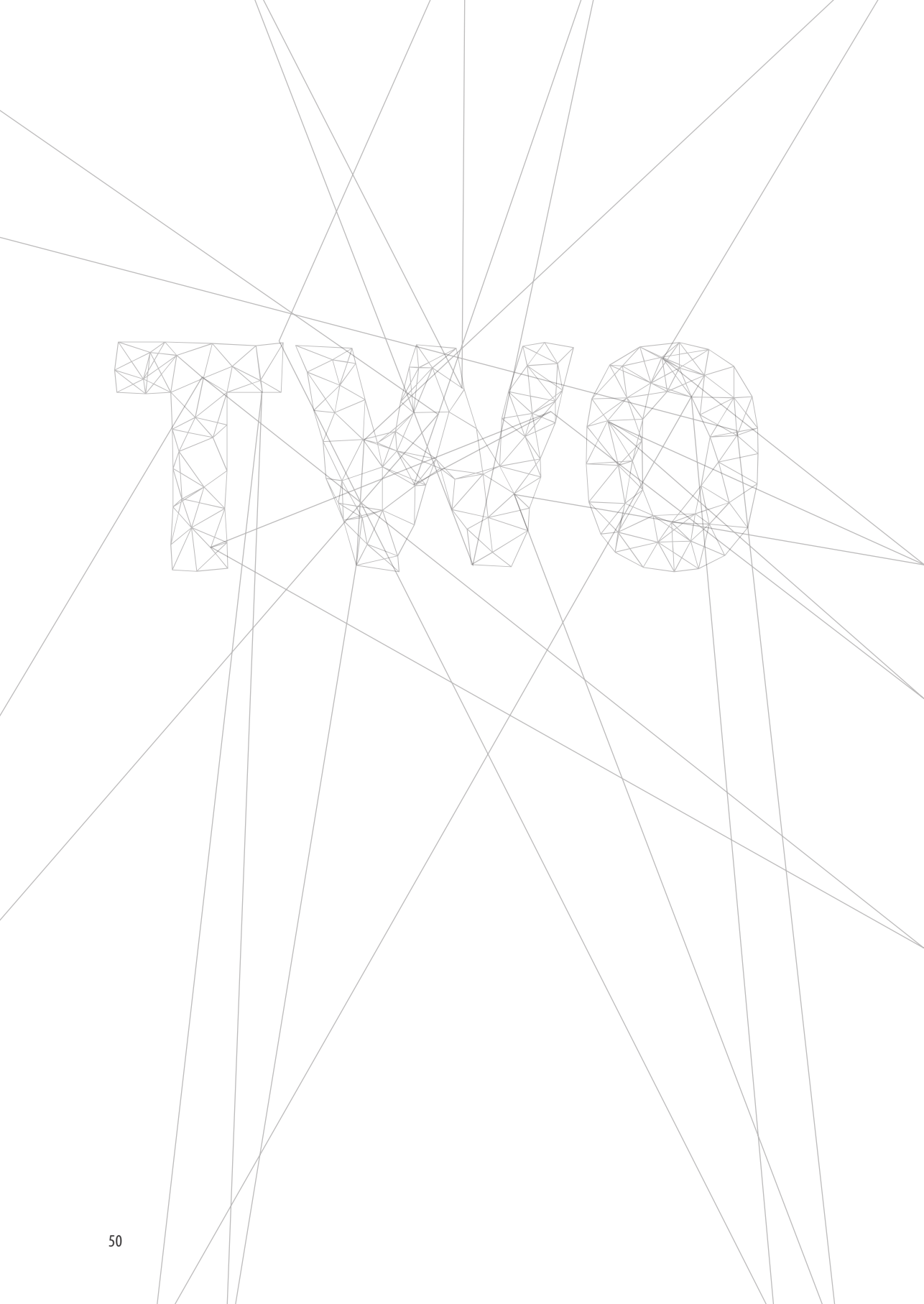
TWO

Chapter 2

Combined DNA extraction and antibody elution from filter papers for the assessment of malaria transmission intensity in epidemiological studies

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Malaria Journal. 2013;12(1):272

Abstract

Background

Informing and evaluating malaria control efforts relies on knowledge of local transmission dynamics. Serological and molecular tools have demonstrated great sensitivity to quantify transmission intensity in low endemic settings where the sensitivity of traditional methods is limited. Filter paper blood spots are commonly used a source of both DNA and antibodies. To enhance the operational practicability of malaria surveys, a method is presented for combined DNA extraction and antibody elution.

Methods

Filter paper blood spots were collected as part of a large cross-sectional survey in the Kenyan highlands. DNA was extracted using a saponin/chelex method. The eluate of the first wash during the DNA extraction process was used for antibody detection and compared with previously validated antibody elution procedures. Antibody elution efficiency was assessed by total IgG ELISA for malaria antigens apical membrane antigen-1 (AMA-1) and merozoite-surface protein-1 (MSP-142). The sensitivity of nested 18S rRNA and cytochrome b PCR assays and the impact of doubling filter paper material for PCR sensitivity were determined. The distribution of cell material and antibodies throughout filter paper blood spots were examined using luminescent and fluorescent reporter assays.

Results

Antibody levels measured after the combined antibody/DNA extraction technique were strongly correlated to those measured after standard antibody elution ($p < 0.0001$). Antibody levels for both AMA-1 and MSP-142 were generally slightly lower (11.3-21.4 %) but age-seroprevalence patterns were indistinguishable. The proportion of parasite positive samples ranged from 12.9 % to 19.2 % in the different PCR assays. Despite strong agreement between outcomes of different PCR assays, none of the assays detected all parasite-positive individuals. For all assays doubling filter paper material for DNA extraction increased sensitivity. The concentration of cell and antibody material was not homogenously distributed throughout blood spots.

Conclusion

Combined DNA extraction and antibody elution is an operationally attractive approach for high throughput assessment of cumulative malaria exposure and current infection prevalence in endemic settings. Estimates of antibody prevalence are unaffected by the combined extraction and elution procedure. The choice of target gene and the amount and source of filter paper material for DNA extraction can have a marked impact on PCR sensitivity.

Background

To effectively implement and evaluate malaria control efforts a detailed knowledge is required of *Plasmodium* carriage and transmission within target populations. Transmission intensity is traditionally assessed using mosquito trapping techniques to determine exposure to infected *Anopheles* mosquitoes. In low endemic areas, where vector populations may be sparsely infected, small or heterogeneously distributed, trapping becomes operationally and technically unattractive [1-3]. A frequently used alternative is the prevalence of malaria infection in human populations, which is typically assessed by light microscopy. However, the limited detection limit and operational constraints of microscopical surveillance present a major barrier to its application in low endemic areas [4-8]. With patterns of reducing malaria transmission intensity in many African settings [9-14], it will become increasingly important to have sensitive alternatives for population level surveillance in areas approaching a phase of elimination [7, 15].

Serological and molecular tools have been proposed to be particularly useful for monitoring transmission intensity and determining parasitaemia among populations in areas of low endemicity. Antibody responses to recombinant asexual malaria antigens are strongly associated with entomological measures of transmission intensity and microscopical parasite prevalence [16], but at low endemicity have a greater discriminative power [3]. Low level transmission may be detectable in the absence of microscopically detectable infection [17] and serological markers can detect spatial variation in transmission intensity [18] and the efficacy of interventions [19].While serology can be used to detect spatial and temporal patterns in transmission intensity [20], antibody responses are long-lived and, unless sampling is restricted to very young age groups, additional tools are required to quantify on-going transmission. The polymerase chain reaction (PCR) is a highly sensitive method for detecting *Plasmodium* infection at all levels of endemicity [21-23]. In a meta-analysis comprising 106 surveys, microscopy detected 54.1 % of all PCR-detected infections; a figure that decreased to below 20 % in low endemic settings [24]. Sub-microscopic parasite carriage has been shown to contribute significantly to the malaria infectious reservoir [25, 26] and is therefore of relevance for inclusion in control programmes. Actively identifying infected individuals using PCR may, therefore, be critically important when attempting to interrupt malaria transmission [7, 27, 28]. While PCR is commonly used as gold standard for detecting all parasitaemic individuals, there is variation between different PCR approaches [29, 30] and DNA extraction from filter papers may vary in efficiency [30, 31].

In the context of malaria elimination, there is a need to optimize molecular and serological assays for rapid and simultaneous assessment of the significant numbers of samples that will be generated by large scale, long term surveillance [32]. At present, DNA extraction and antibody elution are the most time consuming and laborious aspects of serological and molecular assessments. It would be operationally attractive to source DNA

and antibodies from the same blood spots, as this would allow serology and PCR to be conducted in unison, increasing throughput while decreasing costs.

Here, a simple method for concurrently extracting antibodies and DNA from filter paper blood spots is presented. Antibody responses to malaria antigens are assessed to compare the efficacy of antibody elution. PCR assays using two different target genes are compared, andtwo sources of variation in PCR outcome are explored: the distribution of parasite material on filter papers and the amount of filter paper material that is used for DNA extraction.

Methods

Study area and subjects

Blood spot samples were collected as part of a cross-sectional study in the Western Kenyan Highlands (latitude -0.470431°, longitude 34.842628°) in June 2011 that received ethics approval from Scientific Steering Committee (SSC), the Ethical Review Committee (ERC) of the Kenya Medical Research Institute (KEMRI) Nairobi (proposal numbers SSC 2163, 2181 and 1589), the London School of Hygiene & Tropical Medicine ethics committee (#6111), and from Centers for Disease Control and Prevention (with exempt status) [33]. Blood derived from a finger prick was blotted onto Whatman no. 3 filter paper (Whatman, Maidstone, UK) and was dried overnight before storage with silica gel at -20 °C. Each filter paper contained three individual blood spots of indeterminate volume. Filter papers were wetted through by blood spots completely, as described by Corran et al [34]. A subset of 240 randomly selected blood spots was selected for both PCR and enzyme-linked immunosorbent assay (ELISA).

Standard antibody elution

The full protocol of the elution and extraction steps is provided in the supporting documentation (Additional file 1). Three filter paper discs of 2.5 mm in diameter were punched from the centre of each dried blood spot. Filter paper discs were immediately placed into the wells of replicate 2.0 ml 96 deep well plates (Axygen Biosciences, CA, USA), one containing individual discs, the other pairs of discs. Each plate contained 80 samples so that the sample number in each corresponded to that of 2 ELISA plates, leaving wells free for controls. For standard elution single filter papers were incubated in 1120 μl of a 0.5 % sodium azide/ PBS solution [34]. Plates were sealed and placed onto a plate shaker on their side, allowing the cut filter paper discs to move freely along the length of their wells. After overnight incubation, the eluate was stored at -80 °C. The final serum dilution of the eluate based on estimates of the volume of whole blood in a 2.5 mm filter paper disc was 1:400 [34].

Combined antibody elution and DNA extraction

Filter paper discs were prepared and stored in deep well plates as for standard antibody elution. For combined DNA extraction and antibody elution (henceforth combined elution), 1120 µl of a 0.5 % saponin/PBS solution was added to each well and plates were incubated overnight as for standard elution. 200 µl of the eluate, which contained all soluble elements including antibodies, was transferred to a new plate and stored at -80 °C until use in ELISA. To continue with DNA extraction, the remaining saponin solution was aspirated and 1 ml of PBS washing solution was added to each well at 4 °C. Plates were horizontally incubated on a shaker for one hour as above, before PBS was aspirated and discarded. 150 µl of a 6 % Chelex in DNase/ RNase free water solution was added to each sample. Plates were sealed using adhesive foil mats (Axygen Biosciences, CA, USA) and incubated in a water bath for 3*10 minutes at 97 °C. Between 10 minute incubations plates were briefly centrifuged in order to relieve pressure and ensure optimal DNA elution. After the last incubation plates were spun down at maximum speed for 5 minutes to allow the Chelex to settle. 120 µl of the DNA containing solution was taken and aliquoted into new plates. Samples were stored at -80 °C until further analyses. To exclude the risk of cross-contamination during extraction materials were extensively tested using positive and negative controls (2.5 % *Plasmodium* DNA and blank wells respectively). No cross-contamination was observed during extraction.

AMA-1 and MSP-142 ELISA

IgG antibody responses against AMA-1 (BPRC, 0.3 µg/ml coating concentration) and MSP-142 (FVO, 0.2 µg/ml coating concentration) were detected as previously described [16, 35]. Test sera were analysed in duplicate at 1:1000 (MSP-142) or 1:2000 (AMA-1) in PBST/Marvel milk powder (Cadbury, UK). Blank wells and a serial dilution of pooled hyper-immune sera were included in duplicate on each plate to correct for non-specific antibody reactivity and standardise responses for inter-plate variation. Seroprevalence of IgG antibodies to both antigens was determined using a mixture model as described previously [16, 34]. The model was used on each population of samples, giving four separate positivity thresholds (one each for AMA-1 standard elution, AMA-1 combined elution, MSP-142 standard elution, and MSP-142 combined elution).

Parasite detection by PCR

Three nested PCR assays were evaluated; an 18S PCR targeting the small ribosomal subunit of *Plasmodium falciparum* developed by Snounou et al [22] and two variations of a more recent assay which targets the mitochondrial cytochrome b as described by Steenkeste et al. [36, 37]. Because of inconsistent amplification of amplicons generated by the nest 1 (N1) primers described by Steenkeste et al primers of the N1 reaction were redesigned. The 18S PCR was performed according to the original protocol except that the quantity of template used in the N1 reaction was increased from 1 µl to 5 µl. In every set of PCR conditions 5

μL template was used in the N1 reaction and 1.5 μl of product in the N2 reaction. For a more detailed overview of primer sequences, product sized and PCR cycling conditions see Additional file 1 Pooled DNA extracts from *P. falciparum* NF54 cultured in Nijmegen, the Netherlands were run on every PCR plate as a positive control, alongside a negative water control. Positive control was diluted to the extent that both N1 and N2 fragments were sufficiently amplified so that both amplicons could be visualized on gel. N1 and N2 products were mixed and 10 μl was visualized on a 0.8 % agarose gel by electrophoresis in 0.5 x Tris-acetate-EDTA buffer (0.04 M Tris-acetate and 1 mM EDTA, pH 8.0). Each assay was assessed using single and double filter papers, creating a total of 6 PCR conditions for comparison.

Distribution of parasite material on filter papers

To visualize cell material in filter paper blood spots two C57BL/6 mice were infected as previously described with a transgenic *Plasmodium* berghei strain (PbGFP-Luccon) expressing a fusion protein of GFP and Luciferase from the eef1a promoter [38, 39]. The original studies that were used as a source of blood material were performed according to the regulations of the Dutch "Animal On Experimentation act" and the European guidelines 86/609/EEG; approval was obtained from the Radboud University Experimental Animal Ethical Committee (RUDEC 2009-019). 100 μl of blood from the infected mice was collected in heparinised tubes and mixed with 3.2 μl of highly concentrated (67 mg/ml) D-luciferin (Xenogen, CA, USA) dissolved in PBS [38]. 30 μl of this mixture was pipetted onto Whatman no. 3 paper (Whatman, Maidstone, UK) in a manner closely approximating that of blood spot collection in the field. Drops were first formed on the pipette tip before contact with the paper was made, and filter papers were wetted through completely. Blood spots were left to dry for 15 minutes before luminescent imaging was performed using a Lumina Caliper (PerkinElmer, MA, USA) (5 cm FOV, medium binning factor, 1 second exposure). This process was repeated for two blood spots. A blood spot without the addition of D-luciferin was used as a negative control.

Distribution of antibody material on filter papers

Batches of 100 μl whole human blood were mixed with fluorescent labeled anti- APC-Cy7-anti-CD4 (Biolegend, CA, USA) and/or anti-human APC-IL-2. (eBioscience, CA, USA). Blood spot preparation and imaging was performed as in the cell distribution experiments.

Data analysis

Statistical analysis was conducted using STATA 12 (StataCorp., TX, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). IgG responses between groups of paired data were compared by Wilcoxon signed rank test. Seroprevalence comparisons were made using Chi-square test, with a test for trend in proportions. Associations between IgG

responses expressed as antibody titre were quantified by Spearman correlation coefficients, and differences between elution approaches tested by linear regression presenting 95% confidence intervals (CI). The level of agreement, kappa value and sensitivity were assessed by comparing individual PCR conditions with 'true positivity' that was defined as positivity in any one of the PCR assay variants. The difference in the proportion of positive samples between PCR conditions was tested by McNemar's chi-square for paired data. To minimize the influence of possible false positive PCR results on sensitivity estimates, calculations were repeated after 'true positivity' was defined as a positive PCR in at least two of the PCR conditions. For the cell/antibody distribution experiments Living image 3.2 was used (PerkinElmer, MA, USA). To map the relative fluorescence/intensity in different areas of the blood spot they were overlaid with grids containing cells of 2.5 mm2. Grids extended to the spots edges, and cells were excluded from analysis if their area was not entirely filled with dried blood. For comparison between cells, fluorescence and luminescence values were calculated as a proportion of the highest cell value.

Results

Antibody responses

Antibodies were eluted from 236 filter papers by both the standard elution procedure and the combined elution procedure. For both AMA-1 and MSP-142, a strong positive correlation was observed between the IgG responses of filter paper blood samples eluted by standard and combined methods (Figure 1). While strongly correlated, there was a tendency toward higher antibody responses when samples were eluted using the standard methodology for both antigens ($p=<0.0001$). For antibody level (optical density), responses were on average 11.3% higher for AMA-1, and 21.4% higher for MSP-142 when using standard rather than combined elution. Linear regression analysis showed that for AMA-1 an increase of titre 1 using combined elution was associated with an increase of titre 1.773 (95 % CI 1.712-1.834; $p<0.0001$) using standard elution. For MSP-142 an increase of titre 1 using combined elution was associated with an increase of titre 1.811 (95 % CI 1.647-1.975, $p<0.0001$) using standard elution. For use as marker of exposure, antibody responses against AMA-1 and MSP-142 are commonly combined to give a prevalence of any anti-*P. falciparum* antibodies [17, 18, 20]. Between standard and combined elution methods seroprevalence of antibody responses to AMA-1 and MSP-142 did not differ significantly ($p >0.8$), and for both methods showed a strong age-dependent increase (Figure 2; $p <0.0001$). Within age-groups, antibody seroprevalence did not differ significantly between elution methods ($p >0.5$).

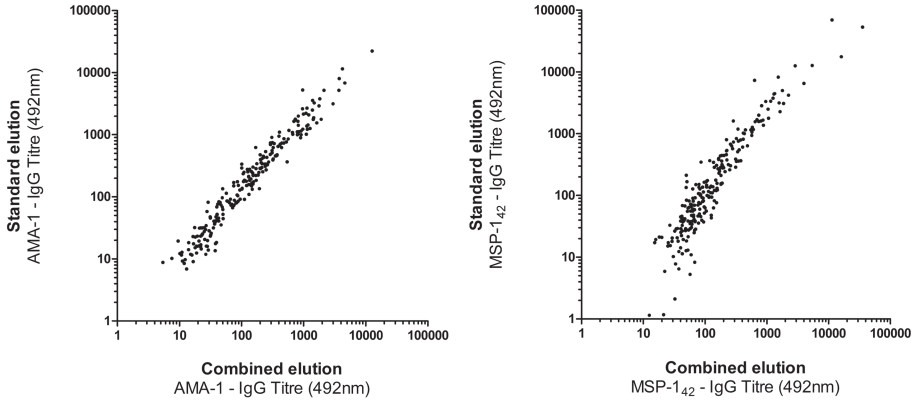

Figure 1. Antibody level from standard and dual filter paper blood spot elution methods for AMA-1 and MSP-142. A. Scatter plot showing anti-AMA-1 IgG level detected in 236 individuals by standard (x-axis) and combined (y-axis) elution of filter paper blood spots. R2 (linear regression) = 0.93 (p=<0.0001). B. Scatter plot showing anti-MSP-142 IgG level detected in 236 individuals using standard (x-axis) and combined (y-axis) elution of filter paper blood spots. R2 (linear regression) 0.671 (p=<0.0001).

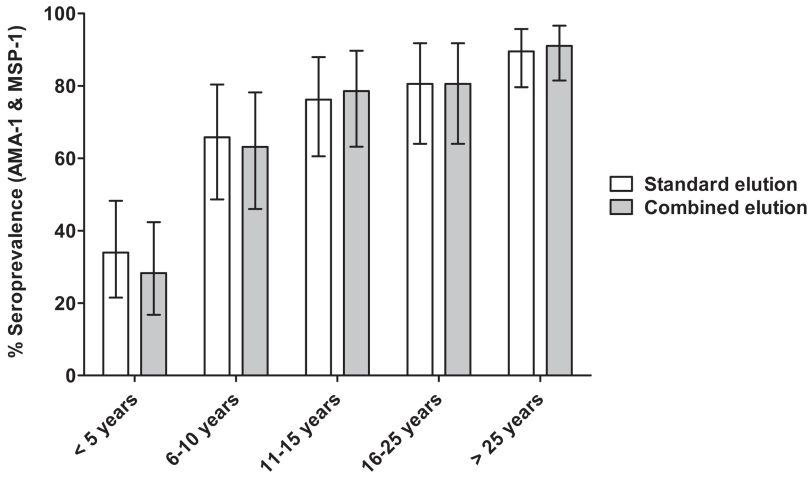

Figure 2. Seroprevalence of anti-AMA-1 or MSP-142 IgG responses by filter paper elution method and age. Error bars indicate 95 % confidence intervals (CI). Sample sizes for the age groups were 53 (< 5 years), 38 (6-10 years), 43 (11-15 years), 36 (16-25 years), and 67 (>25 years).

Table 1. Agreement between 18s, modified cytochrome b and original cytochrome PCR assays

PCR Assay	Filter paper number	Positivity, % (n/N)	Agreement, %	Kappa	Sensitivity, % (95% CI)
18S rRNA	Single	12.9 (31/240)	90.0	0.666	56.4 (42.3 - 69.7)
	Double	16.7 (40/240)	93.8	0.804	72.7 (59.0 - 83.9)
Modified Cytochrome B	Single	15.4 (37/240)	92.5	0.760	67.3 (53.3 - 79.3)
	Double	18.3 (44/240)	95.4	0.861	80.0 (67.0 - 89.6)
Original Cytochrome B	Single	17.9 (43/240)	95.0	0.847	78.2 (65.0 - 88.2)
	Double	19.2 (46/240)	96.3	0.887	83.6 (71.2 - 92.2)

The agreement, kappa value and sensitivity were calculated by comparing individual PCR conditions with 'true positivity' that was defined as positivity in any one of the PCR assay variants. The abbreviation n/N indicates PCR positive individuals (n) as a proportion of the total sample size (N).

Table 2. Consistency of outcomes in different PCR assays in relation to the amount of filter paper material used for extraction

Filter paper number	Never positive (%)	Positive in 1/3 PCR assays (%)	Positive in 2/3 PCR assays (%)	Positive in 3/3 PCR assays (%)
Single	80.8 (194/240)	2.9 (7/240)	5.4 (13/240)	10.8 (26/240)
Double	79.6 (191/240)	1.3 (3/240)	4.6 (11/240)	14.6 (35/240)

The proportion (n/N) of positive PCR assays when aliquots of the same extracted material was used in three different PCR assays.

Parasite prevalence by PCR

Parasite prevalence by PCR differed between different methodologies and ranged from 12.9 % (31/240) when the 18s rRNA-based PCR was used with single filter paper discs to 19.2 % (46/240) when the original cytochrome b based PCR was used with two filter paper discs (Table 1). The level of agreement between these two estimates, representing the two extremes of parasite prevalence, was high (90.4 %, kappa 0.65) but the cytochrome b PCR with two filter paper discs resulted in significantly more positive results compared to the 18s rRNA based PCR with single filter paper discs (p=0.002). When true positivity was defined as a sample being positive in any one of the assays, the standard cytochrome b assay using DNA from two filter paper discs showed the highest sensitivity (83.6 % C.I. 71.2-92.2 %). Defining true positivity as a positive signal in at least two of the PCR assays did not change the estimates of sensitivity and kappa considerably (see Additional file 2).

Doubling filter paper material for DNA extraction increased the sensitivity of PCR assays by 5.4-16.3 %. When pairs of results from the same PCR assay but using DNA template from single or double filters were compared (n=720 pairs), the latter resulted in significantly more parasite positive results (p=0.013). 130 of the PCRs performed on DNA from double filter paper punches were PCR positive, compared to 111 of the PCRs performed on DNA from

single filter paper punches. Surprisingly, 18.0 % (20/111) of the samples that were positive in a PCR using template from a single filter punch were negative in the PCR performed when two punches were taken from the same filter paper.

Doubling filter paper material also appeared to increase the consistency of PCR outcomes on the same DNA material, albeit not statistically significant. When the 18s rRNA-based PCR and both cytochrome b based PCRs were performed on material from the same extraction, inconsistent results (i.e. one or two but not all three PCR assays giving amplification) were observed for 8.3% (20/240) of the samples when material from single filter paper punches was used compared to 5.9% (14/240) of samples when filter paper material was doubled (Table 2, p=0.36).

Distribution of parasite material on blood spots

Luminescence produced by GFP expression in Pb-GFPluccon infected blood samples was previously shown to correlate strongly with parasitaemia [40]. Here, the distribution of DNA on filter paper was assessed by measuring the luminescent intensity in dried blood spots from mice infected with Pb-GFPluccon [41]. Cell by cell luminescence analysis of the grid overlaying the blood spot was used to describe heterogeneity in parasite material in different parts of the blood spot. Both blood spots tested showed a considerable degree of heterogeneity in the distribution of parasite material (Figure 3). In the two separate experiments, 25 % and 64 % of the grid cells contained less than 85 % of the parasite material of the grid cell with the highest quantity. Parasite material seemed less concentrated towards the extreme edges of the blood spot; the grid cell with the lowest parasite quantity contained 70% of the maximum value (Figure 3).

Distribution of antibody material on blood spots

The distribution of antibodies on filter paper was evaluated by the adding two marker molecules which have approximately the same molecular weight of human IgG and have no detectable interaction with other components in human blood. Analysis of fluorescence intensity based on the overlaying grid showed that also antibodies were heterogeneously distributed throughout the blood spot (Figure 3). In the two separate experiments, 65 % of the grid cells contained less than 85 % of the parasite material of the grid cell with the highest quantity. Contrary to the observations on parasite material, there was no evident pattern in antibody concentration on the blood spot and concentrations of antibodies did not appear to be higher in the middle of the spot (Figure 3). The grid cell with the lowest antibody concentration contained 67% of the maximum value.

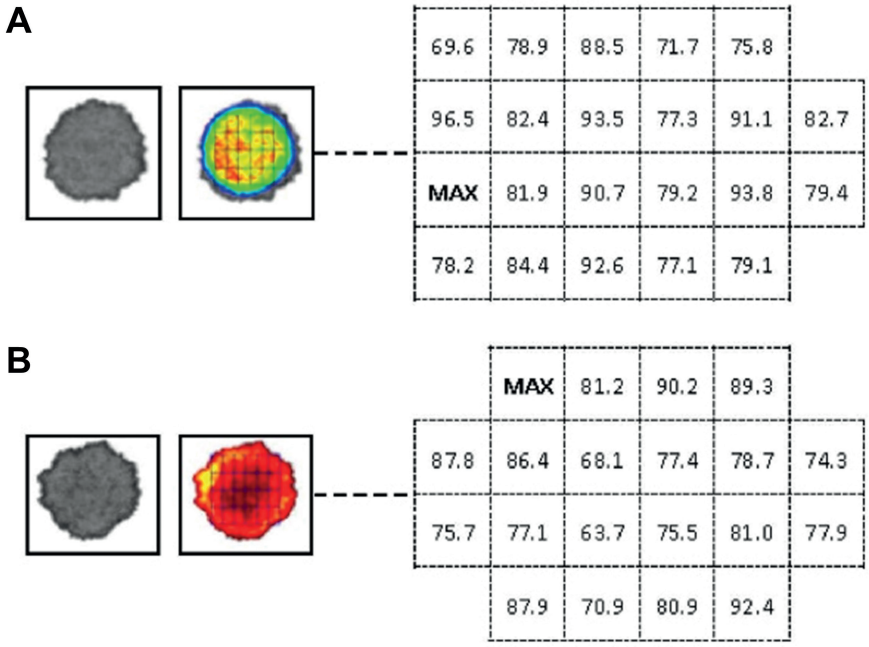

Figure 3: DNA/Cell and antibody distribution on filter paper. A. Figure 3: DNA/Cell and antibody distribution on filter paper. A. Photograph of one of the two blood spots analysed for cell material distribution, in which luminescence intensity is proportional to the density of cell material. Luminescence intensity values in the cells of the overlying grid are calculated as a proportion of the highest cell value, and are presented in the adjacent grid schema. B. Photograph of one of the two blood spots analysed for antibody material distribution, in which fluorescence intensity is proportional to the density of antibodies. Fluorescence intensity values are calculated and presented as in Figure 3A.

Discussion

The methodology described in this report offers a cost-efficient high-throughput approach to preparing large numbers of filter paper samples for the assessment of cumulative malaria exposure and current infection status. Used in concert, serological and molecular assays can provide detailed insight into the transmission dynamics of *Plasmodium*.

The utility of serological assessments in malaria surveillance has been evidenced by numerous studies of the antibody responses of endemic populations to recombinant *Plasmodium* [42] and *Anopheles* [43-45] antigens. Recently the importance of molecular tools in malaria surveillance has been emphasised, as it has become apparent that the extent and relevance of sub-microscopic malaria infections in low endemic areas may be much greater than previously assumed [6, 24]. As the number of areas making efforts to reduce or

interrupt native malaria transmission grows so will the importance of sensitively detecting malaria exposure [7, 32, 46]. As such, the development of strategies to ease sample collection and processing during wide-scale population level surveillance is both timely and apposite to the wider malaria eradication agenda. The use of filter papers for blood collection and their subsequent storage and processing for sero-epidemiological analyses was discussed in depth by Corran et al. [34]. Since this time many studies have benefitted from the use of filter paper blood spots as a source of serum antibodies to reveal age-dependent [20], spatial [17-19] and temporal [47] patterns in cumulative malaria exposure. In the current study antibody levels (OD) from the standard elution methodology and the combined elution methodology (in which a portion of the filter paper eluate undergoes onward processing for DNA extraction) show a strong and highly significant correlation. Though the relationship between absolute antibody titre in paired measures was strongly related, higher antibody levels were generally observed when blood spots underwent standard elution procedures. The reason for this is unknown and may reflect differences in the relative concentration of detergent. The lower antibody yield in the combined method warns against using the two approaches simultaneously; quantitative outcomes of individual samples cannot be directly compared when different elution methods have been used. In epidemiological studies it is more common to analyse variation in malaria exposure using measures of (age-dependent) antibody seroprevalence [19, 20, 48]. In the current study, seroprevalence did not differ significantly between the two elution methods, and both methods showed the same age-dependent acquisition of antibody responses. This indicates that combining antibody elution with DNA extraction is an operationally attractive alternative to the standard method of antibody elution that can reliably be used to compare antibody responses between populations of blood donors.

The elution of antibodies during the process of DNA extraction adds an advantage to the chelex/saponin extraction method, which is probably the most widely used extraction method in epidemiological malaria studies. This extraction method has repeatedly been shown to give comparable results when compared to commercial extraction kits [49, 50]; although in case of older or incorrectly dried and stored filter papers commercial kits may be recommended [31]. Because of its evident superior sensitivity compared to microscopy [6, 24], PCR may be considered to be the gold standard for the detection of malaria infections in epidemiological studies. The current study highlights three relevant caveats to this assumption. Firstly, different PCRs differ in their sensitivity to detect malaria parasite. Although a recent meta-analysis found no differences in sensitivity compared to microscopy for different nested PCR assays [24], the current study presents evidence for a higher sensitivity of PCR based on the cytochrome b target gene compared to the most widely used 18s rRNA target gene [31, 51]. This may be due to better conservation of mitochondrial material [52]. The current study shows no advantage of the newly designed primers for the cytochrome b gene in overcoming the anecdotal problems of inconsistency

of PCR results with the original protocol. The shorter N1 primers amplify the same area , may give more consistent results (data not shown) and may increase primer stability during freeze/thawing cycles but did not lead to improved sensitivity for the 240 samples tested in the current study.

Secondly, the current study presents evidence that increasing the amount of filter paper material for DNA extraction results in an increased sensitivity. Although this finding is intuitively correct, its actual relevance for determining parasite prevalence in field studies has not been described in detail before. One could argue that a single copy of template material may result in successful amplification and therefore only infections with densities close to the threshold density for detection by PCR would give discordant PCR results. The current findings suggest that this is frequently the case and that doubling filter paper material can lead to parasite prevalence estimates that are up to 3.8 % higher.

Thirdly, the results illustrate the stochastic nature of PCR. Although the agreement between PCR outcomes was very high, agreement was never perfect. Discordant PCR results were common, especially if single filter paper punches were used. Importantly, some PCRs performed on single filter paper punches detected parasites while the same PCR on double filter paper punches did not. This serves as a word of warning against assuming 100 % sensitivity of PCR. It has been previously acknowledged that PCR assays may fail to detect all circulating parasite clones [53, 54], the current study indicates that this imperfection of PCR assays may also affect parasite prevalence estimates. The exploratory experiments described in this study on the distribution of parasite material on filter papers may be relevant in this respect: the amount of parasite material can differ by more than 15% between different punches from the same bloodspot despite the blood completely wetting the paper.

Conclusion

When the combined DNA extraction-serum elution methodology is used, robust PCR and ELISA results can be obtained. The combined approach can significantly reduce the workload in large-scale epidemiological studies and allow efficient use of blood spot material for molecular and immunological assays. The efficient use of blood spot material may allow researchers to increase the amount of filter paper material that is used for this combined extraction. This will increase PCR sensitivity and may increase robustness of parasite prevalence estimates.

acknowledgments

We thank project staff, the community of Kabondo and Kasipul, Rachuonyo South, and KEMRI/CDC Kisumu. In addition we would like to thank Patrick Corran for his advice on the filter paper antibody distribution experiments. We would also like to thank Alanna Swartz and Bryan Greenhouse for the fruitful discussions on this subject. This project was

funded by the Bill and Melinda Gates Foundation as part of a Grand Challenge project (Grant No. OPP1024438) and the Malaria Transmission Consortium (Grant No. 45114). This manuscript was submitted for publication with approval of the director of the Centre for Global Health Research, Kenyan Medical Research Institute, Kisumu.

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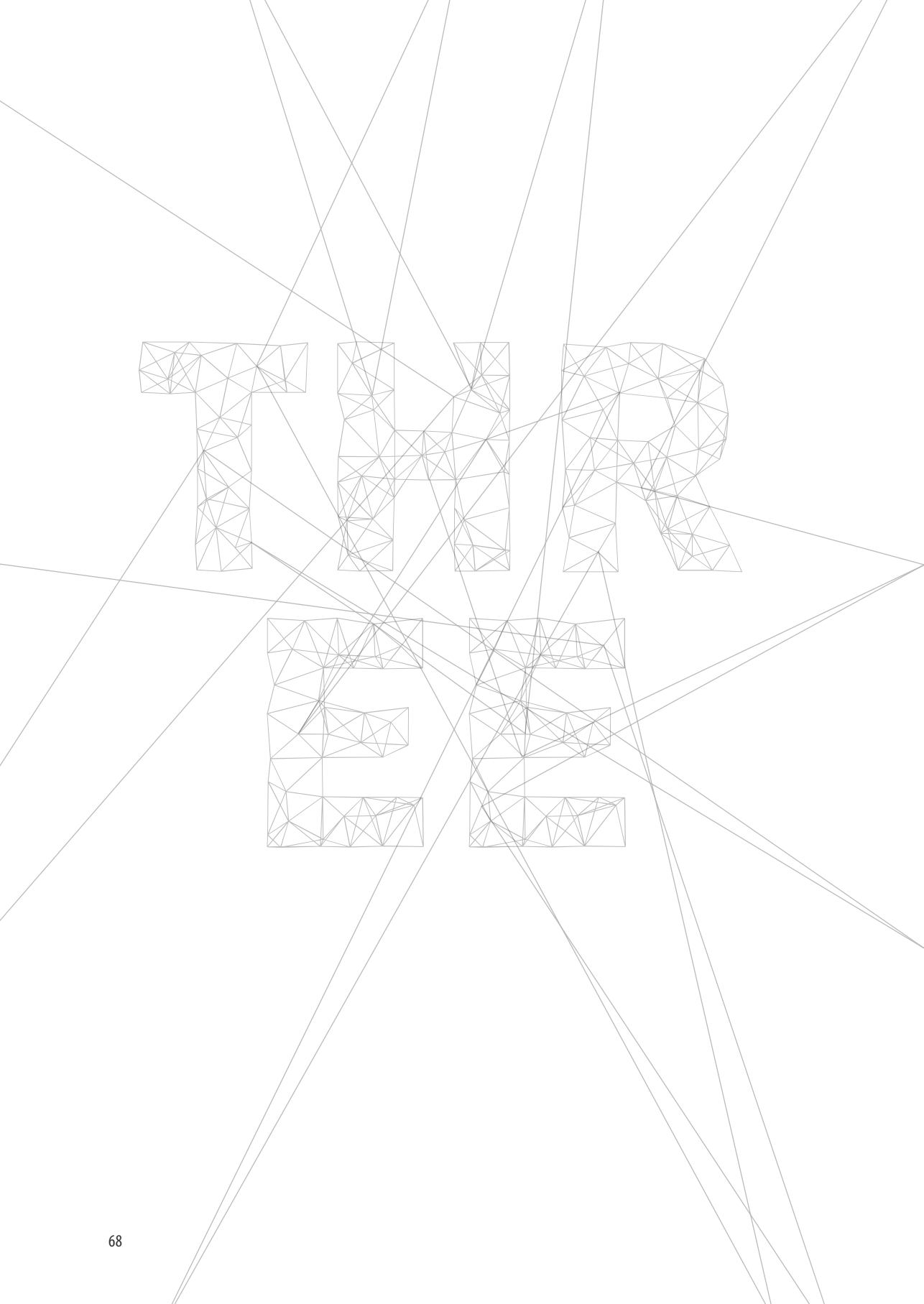
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Chapter 3

IgG responses to *Anopheles gambiae* salivary antigen gSG6 detect variation in exposure to malaria vectors and disease risk

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PLoS ONE. 2012;7(6):e40170

Abstract

Assessment of exposure to malaria vectors is important to our understanding of spatial and temporal variations in disease transmission and facilitates the targeting and evaluation of control efforts. Recently, an immunogenic *Anopheles gambiae* salivary protein (gSG6) was identified and proposed as the basis of an immuno-assay determining exposure to Afrotropical malaria vectors. In the present study, IgG responses to gSG6 and 6 malaria antigens (CSP, AMA-1, MSP-1, MSP-3, GLURP R1, and GLURP R2) were compared to *Anopheles* exposure and malaria incidence in a cohort of children from Korogwe district, Tanzania; an area of moderate and heterogeneous malaria transmission. Anti-gSG6 responses above the threshold for seropositivity were detected in 15% (96/636) of the children, and were positively associated with geographical variations in *Anopheles* exposure (OR 1.25, CI 1.01–1.54, p=0.04). Additionally, IgG responses to gSG6 in individual children showed a strong positive association with household level mosquito exposure. IgG levels for all antigens except AMA-1 were associated with the frequency of malaria episodes following sampling. gSG6 seropositivity was strongly positively associated with subsequent malaria incidence (test for trend p=0.004), comparable to malaria antigens MSP-1 and GLURP R2. Our results show that the gSG6 assay is sensitive to micro-epidemiological variations in exposure to *Anopheles* mosquitoes, and provide a correlate of malaria risk that is unrelated to immune protection. While the technique requires further evaluation in a range of malaria endemic settings, our findings suggest that the gSG6 assay may have a role in the evaluation and planning of targeted and preventative anti-malaria interventions.

Introduction

Heterogeneity in malaria exposure is present at all levels of endemicity [1] but is most readily observed in areas of low transmission and following periods of extensive control [1-3]. Recent evidence of decreasing malaria incidence [2,4], has fuelled calls for malaria elimination from the world's public health, political and philanthropic authorities [5,6]. As a result the interest in malaria heterogeneity and its potential effect on malaria control has increased [2,3,7]. Hotspots of higher malaria transmission are likely to hamper malaria elimination efforts, as residual foci of persistent malaria infection may seed transmission to the wider community [8-10].

Although not all factors that affect malaria heterogeneity are fully understood, variation in the exposure to malaria vectors is likely to be of key importance [3,11-13]. In sub-Saharan Africa, the transmission of *Plasmodium falciparum* is maintained by three key mosquito species; *Anopheles gambiae*, *An. arabiensis* and *An. funestus* [14]. Mosquito exposure is typically assessed as a component of the entomological inoculation rate (EIR), which is defined as the number of infectious *Anopheles* bites per person per unit time (ib/p/yr) [15,16]. Despite its value in malaria research, a direct assessment of EIR to determine small-scale variation in malaria exposure is operationally unattractive at low levels of transmission (EIR<10 ib/p/yr) [17-19]. The development of accurate and sensitive tools for identifying micro-epidemiological variations in vector exposure and malaria risk is important in assessing the efficiency of control efforts and focusing interventions to those areas or populations that are most affected by malaria. Serological assessments of malaria exposure are receiving increasing interest in this respect and have been used for quantifying malaria transmission intensity [20] and its temporal [21] and spatial variation [11,22,23]. Recently, serological markers of malaria exposure were also used to quantify heterogeneity in the efficacy of malaria interventions [24]. Recombinant malaria blood stage antigens have been most widely used for these purposes [25], while responses to the infective sporozoite specific circum-sporozoite protein (CSP) are currently viewed as the best available serological tool to detect exposure to infectious mosquito bites [18,26-28]. A similar tool to identify spatial patterns of cumulative exposure to *Anopheles* biting could be integral to the detection of malaria hotspots and play a role in forecasting the risk of malaria epidemics or the dynamics of malaria resurgence in areas where parasite carriage in human populations has decreased but exposure to malaria vectors persists [29].

Our understanding of the human immune response to mosquito saliva has until recently been largely restricted to culicine mosquitoes and the clinical consequences of allergy [30-32]. Humoral responses to the saliva of various disease vectors have been exploited epidemiologically, revealing significant correlation with disease seropositivity and vector exposure. Such assays have now been described for *Ixodes* ticks [33,34], triatomine bugs [35], *Glossina* tsetse flies [36] and *Lutzomyia* and *Phlebotomus* sand flies [37,38]. Recently,

transcriptome analysis of the salivary glands of *A. gambiae* females identified over 70 putative secreted salivary proteins [39-41]. A small (~10kb) immunogenic protein, *gambiae* salivary gland protein 6 (gSG6), that is well conserved in the three major Afrotropical malaria vectors (*A. gambiae, An. arabiensis* and *An. funestus*) and restricted to anopheline mosquitoes [42], has been identified as a suitable candidate for a bioassay of *Anopheles* exposure [43,44]. Antibody responses to a gSG6 peptide (gSG6-P1) described *Anopheles* exposure in areas of low vector density [45] and in response to vector control programs [46] with some success, and were recently shown to reflect *Anopheles* heterogeneity at the district level in Dakar, Senegal [47]. Recombinant full length gSG6 has also shown strong immunogenicity among rural populations in Burkina Faso, which appears to be sufficiently short lived to correlate with seasonal changes in *Anopheles* abundance [43,48]. The relationship between malaria case incidence and anti-gSG6 response has not been studied, despite early indications that humoral responses to *Anopheles* whole saliva were positively associated with malaria infection [49].

Using a subset of samples collected during a large study of intermittent presumptive treatment among infants (IPTi) [50], along with entomological data from an intensive survey in the same area [11], we present the first evaluation of IgG antibody responses to the recombinant gSG6 salivary antigen for describing spatial heterogeneity in vector exposure between and within geographically defined subvillages in an area of moderate and heterogeneous malaria exposure in northern Tanzania. At the individual level, we determine the association of gSG6 reactivity with household *Anopheles* exposure and subsequent malaria incidence. In addition, we determined reactivity against a selection of malaria antigens that have been more commonly used in epidemiological studies, namely CSP and four blood stage proteins, AMA-1, MSP-1, MSP-3, and glutamate-rich protein (GLURP).

Methods

Ethics statement

Witnessed written consent was provided by the caregivers of all children involved in serological sampling, and by heads of households for participation in the entomological survey. Ethical approval was granted by the review board of the National Institute for Medical Research of Tanzania, and the London School of Hygiene and Tropical Medicine ethics committee.

Study area and subjects

Plasma samples were collected from children recruited over 18 months, as part of a longer term study (2004-2008) carried out in the district of Korogwe, Northern Tanzania, an area of moderate malaria endemicity. Korogwe district is situated ~600m above sea level, and

has a seasonal pattern of rainfall (800-1400mm/year) [50]. Malaria transmission in the Korogwe region has declined in recent years [51], such that an EIR of 1-14 ib/p/yr was estimated in 2007 [21]. The original study investigated the relative impacts of different drug regimens for intermittent presumptive treatment (IPTi) among a total of 1280 infants [50].

Entomological data collection

In the final year of the IPTi study a randomly selected subset of 600 children were enrolled in a detailed entomological survey, aiming to describe spatial patterns of malaria incidence in relation to mosquito exposure [11]. In the room of each selected child, mosquitoes were sampled with miniature CDC light traps (Model 512; *John* W. *Hock* Company, Gainesville, Florida) for one night at the end of the wet season (May), again at the beginning (July) and finally the end (September) of the dry season in 2008. Mosquito exposure at the household level was highly correlated between all surveys (correlation coefficient: May/July=0.462, May/September=0.497, July/September=0.444; $p<0.0001$). Mosquito data from first of the three sampling points, during the peak transmission season when *Anopheles* abundance was highest, was therefore deemed adequate in displaying variation in exposure. Of the total *Anopheles* females caught during sampling, *A. gambiae s.l.* made up 80.3%, *An. funestus* 18.6% and other anophelines 1%.

Clinical data and plasma samples

Malaria incidence was assessed by passive monitoring for signs of illness throughout the 22 months following recruitment, during which time free access to clinical treatment was provided [50]. The average age at recruitment was 9.4 weeks (range 8–17 weeks) and infants were recruited at different times of the year, i.e. at different time-points in the transmission season. Plasma samples used in the current study were taken at 9 months of age when infants were presented at clinics as part of the Expanded Program on Immunisation (EPI). Blood samples were collected by finger prick and after plasma separation samples were stored at -20°C until processing. In our analyses, we included malaria incidence in the period between serum collection at 9 months of age and the end of follow-up. This gave an effective follow up period of approximately 15 months and ensured that the follow-up period included one or more peak malaria transmission seasons for each child. The current analyses are an ancillary study and many of the blood samples had been used previously for other IPTi specific investigations. As a result of this non-systematic exhaustion of samples, sera were available for a subset of 636/1280 children for gSG6 ELISA; 247/636 children from this subset were involved in the household level entomological survey.

gSG6 ELISA

ELISA was performed as previously described with few modifications [43,48]. Briefly, Maxisorp 96-well plates (Nunc M9410) were coated with gSG6 at 5ug/ml. Test and negative control serum were analysed in duplicate at 1:100 in phosphate buffered saline with 0.05% Tween 20 (PBST)/1% skimmed milk powder (Marvel, UK). On every plate blank wells (PBST/Marvel) were included to correct sample ODs for background antibody reactivity, and positive control sera (1:40 in PBST/Marvel) were analysed to allow standardisation of OD values for day-to-day and inter-plate variation. Positive control sera was provided, with consent, by an employee of the London School of Hygiene and Tropical medicine who was exposed weekly to the bites of approximately 50-100 laboratory bred *A. gambiae s.s* (Kisumu strain) during colony feeding.

Sera from 39 Europeans with no recent history of travel to malaria endemic countries were used as negative controls for calculation of IgG seroprevalence. Cut off for seropositivity among samples was determined as the mean OD of the unexposed sera plus 3 standard deviations.

P. falciparum ELISA and Luminex assays

For this analysis, IgG antibody responses were chosen in preference to IgM for their high antigen specificity. IgG antibody responses against CSP (Gennova, 0.009µg/ml), AMA-1 (BPRC, 0.3µg/ml) and MSP-1_{19} (CTK Biotech, 0.2µg/ml) were detected as previously described [20,27]. Test sera were analysed in duplicate at 1:200 (CSP), 1:1000 (MSP-1_{19}) or 1:2000 (AMA-1) in PBST/Marvel. Blank wells, positive control sera from a hyper-endemic region in the Gambia [20], and a serial dilution of pooled hyper-immune sera were included in duplicate on each plate to correct for non-specific reactivity and allow standardisation of inter-plate variation. Seroprevalence of IgG antibodies to these non-salivary antigens was calculated using a mixture model as described previously [20,52].

Recombinant proteins corresponding to the R1, R2 (Central repeat and C-terminal repeat regions of GLURP), and the C-terminal region of MSP-3 [53] were c*oval*ently coupled to carboxylated luminex microspheres according to the manufacturer's protocol and tested as previously described [54]. Cut-off for positivity was calculated as the mean reactivity in malaria non-exposed European individuals plus 2 standard deviations.

Data analysis

To examine the relationship between patterns of gSG6 reactivity and small scale spatial variation in *Anopheles* exposure, antibody responses were described at the level of subvillages, which are defined by their geographical location (*figure 1*) [11]. The average (arithmetic mean) mosquito exposure for each village was used for ranking villages from low to high mosquito exposure; this rank was related to antibody prevalence and mean log 10 adjusted antibody level per subvillage. This enabled analyses relating to geographic

variations in *Anopheles* abundance for all individuals, irrespective of their involvement in the entomological survey (*figure 2*).

For infants for whom both household mosquito data and plasma samples were available, it was possible to investigate associations between *Anopheles* exposure and antibody reactivity against salivary and malaria antigens at an individual level. For this purpose, households were analysed in quintiles of *Anopheles* exposure (*table 1*).

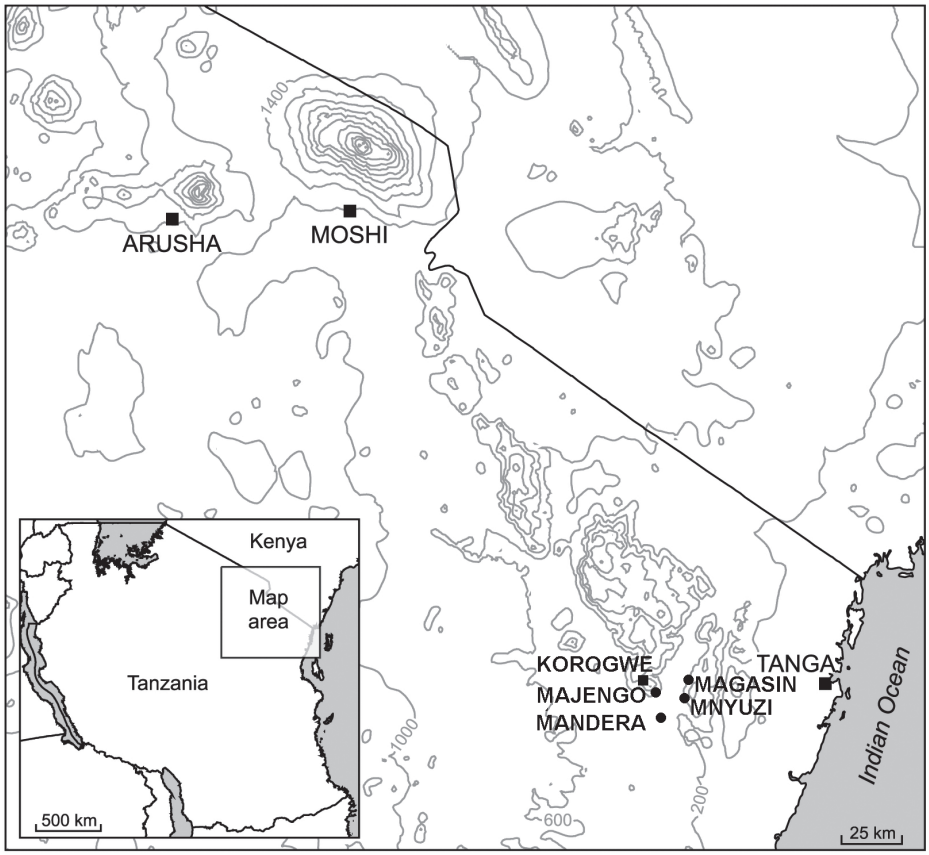

Figure 1. Map of Tanzania showing the north-eastern provinces, and the location of Korogwe district. Sampling in Korogwe district was conducted in 5 areas, which are marked on the map: Korogwe, Majengo, Magasin, Mnyuzi, and Mandera. Within these areas, our study population were resident in 15 subvillages. Korogwe consisted of the following subvillages: Kwasemangube (KS), Lwengera (LW) Msambazi (MS) and Masuguru (MU). Majengo consisted of the following subvillages: Kilole (KI), Majengo (MJ) and Manundu (MA). Magasin consisted of the following subvillages: Kwagunda (KW) and Maguga (MG)**.** Mnyuzi consisted of the following subvillages: Gereza (GE), Lusanga (LU), Mkwakwani (MK), Mnyuzi (MY) and Shambakapori (SH)**.** Mandera (MD) was an isolated subvillage.

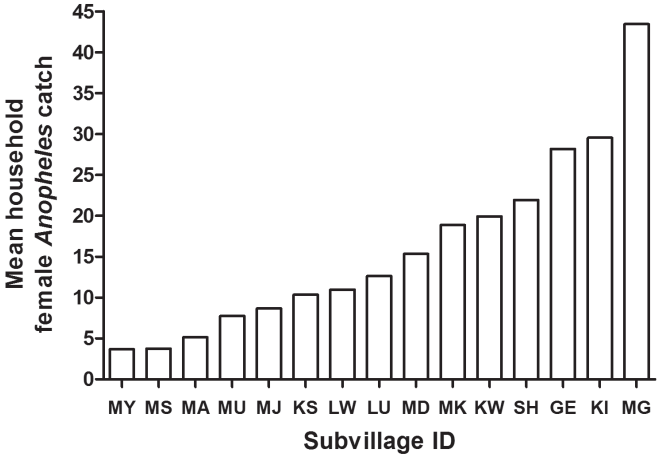

Figure 2. Mean household *Anopheles* female count during peak transmission (May) in different subvillages. Numbers of households sampled for each subvillage, in order of *Anopheles* exposure, were as follows: MY=45, MS=23, MA=26, MU=21, MJ=29, KS=24, LW=65, LU=61, MD=45, MK=14, KW=99, SH=13, GE=47, KI=30, MG=91.

Table 1. Households grouped into quintiles according to their relative exposure to Anopheles females during the wet season entomological survey (May).

Quintile	Households	Female Anopheles per household	
		Mean	Range
1	64	0	0
2	44	1.59	1-2
3	45	4.11	3-5
4	45	11.71	6-17
5	49	43.37	17-119

Table 2. Seroprevalence and IgG antibody levels among seropositive children to *A. gambiae* gSG6, and *P. falciparum* CSP, AMA-1, MSP-1, MSP-3, GLURP R1 and GLURP R2.

	gSG6	CSP	AMA-1	MSP-1	MSP-3	GLURP R1	GLURP R2
Antibody prevalence % (n/N)	15 (96/636)	21 (121/575)	2 (9/540)	10 (52/540)	10 (54/566)	3 (16/566)	12 (67/566)
Median OD (IQR)*	0.29 (0.21-0.58)	0.46 (0.38-0.74)	0.09 (0.07-0.11)	0.21 (0.11-0.33)	-	-	-

OD optical density
IQR inter-quartile range (25th and 75th percentiles)
n/N proportion of seropositive individuals/total sample size
* seropositive individuals only

Statistical analysis was conducted in STATA (Version 10, STATA statistical software StataCorp) and GraphPad Prism (Version 5.0, GraphPad Software Inc., La Jolla, CA) software packages. IgG responses to salivary or malaria antigens between two independent groups were compared by Wilcoxon rank-sum tests (Mann-Whitney U test), with Bonferroni correction for multiple comparisons between subgroups. Comparisons of multiple groups were carried out by Kruskal-Wallis test. Seroprevalence comparisons were made using Chi-square test, with a test for trend in proportions. Correlations between IgG and malaria or entomological measures were made using Spearman correlation or with linear regression analysis after log10-transformation of OD data. IPTi treatment arm was included in our analyses as potential confounder. As a small number of sample ODs were lower than their ELISA plate blank value, some normalised ODs had negative values and an arbitrary positive value (+1) was therefore added to all ODs before transformation.

Results

Small scale spatial variation in Anopheles exposure and anti-gSG6 responses

The recombinant gSG6 protein elicited significant anti-gSG6 IgG responses in children from Korogwe district (mean OD 0.109, maximum OD 2.014). European sera were used as negative controls for exposure to *Anopheles* mosquitoes, the responses of which were pooled to determine a cut-off for seroprevalence at OD 0.167 (*table 2*). Mean OD among antibody negative children from Korogwe was 0.052, and ranged from 0.001-0.166 (standard deviation 0.040). IPTi treatment arm was not associated with gSG6 antibody prevalence (p=0.23) or density (p=0.38) and did not show any evident association with any of the other antigens tested, nor was it found to be a confounder in any of the associations presented below (data not shown).

When mean mosquito exposure was plotted against log 10 adjusted anti-gSG6 IgG level for each of the 15 subvillages, a significant positive association was observed between mean mosquito exposure and antibody reactivity (*figure 3*). Similarly, despite significant variability in gSG6 response between subvillages, there was a significant positive association between mean mosquito exposure per subvillage and anti-gSG6 IgG seropositivity, wherein an average increased exposure of 10 mosquitoes was associated with a 25% increase in antibody positivity (odds ratio [OR] 1.25, CI 1.01–1.54, p=0.04).

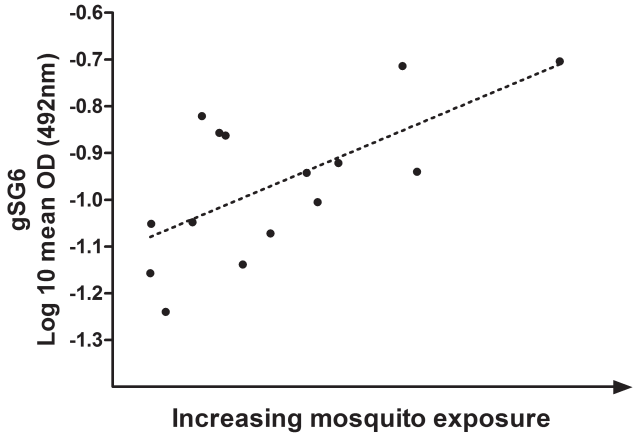

Figure 3. Mean anti-gSG6 IgG level per subvillage, plotted against increasing mosquito exposure per subvillage. Anti-gSG6 IgG levels are given as the log-10 adjusted mean anti-gSG6 OD per subvillage. Mosquito exposure is given as the ascending and sequential mean *Anopheles* female count for each of 15 subvillages (x-axis), as in figure 2. The trend-line from the linear regression is shown as a dashed line (r^2=0.436, p=0.007).

Household level mosquito exposure and anti-gSG6 response

Information on household-level mosquito exposure was available for the households of 247 children. At the level of individual households, exposure to *Anopheles* females showed a significant positive correlation with anti-gSG6 IgG level (correlation coefficient 0.188, p=0.003) but not with levels of anti-CSP IgG (correlation coefficient 0.036, p=0.59). When households were grouped into quintiles according to their relative exposure to *Anopheles* (*table 1*), there was a statistically significant positive association between *Anopheles* exposure in quintiles and anti-gSG6 IgG levels (p=0.001) and prevalence (test for trend in proportions, p=0.001) (*figure 4*). There was no evident association between individual *Anopheles* exposure in quintiles and individual CSP antibody level (p=0.544) or prevalence (test for trend in proportions p=0.422). Similarly, no significant associations were observed between *Anopheles* exposure in quintiles and individual responses to any blood stage antigen, save MSP-3 for which there was a significant positive association with antibody level (p=0.017).

Malaria incidence and anti-gSG6 and anti-malaria responses

Antibody levels were positively associated with the frequency of malaria episodes recorded after serum collection for all antigens except AMA-1 (gSG6 correlation coefficient 0.240, p<0.0001 (*figure 5A*); CSP correlation coefficient 0.183, p=0.004; MSP -1 correlation coefficient 0.256, p<0.0001; MSP-3 correlation coefficient 0.141, p=0.0008; GLURP R1 correlation coefficient 0.126, p=0.003; GLURP R2 correlation coefficient 0.101, p=0.017

[data not shown]). The prevalence of IgG responses varied significantly with grouped malaria incidence for gSG6 (p<0.0001), AMA-1 (p=0.004), MSP-1 (p<0.0001) and GLURP R2 (p=<0.001). No significant variation in seroprevalence of antibodies to CSP, MSP-3 and GLURP R1 was present between groups of malaria incidence (*figure 5B*). A strong positive association was observed between grouped malaria incidence and the prevalence of antibody responses against gSG6, MSP-1 and GLURP R2, while this relationship was present but only marginally significant for MSP-3 (*figure 5B*).

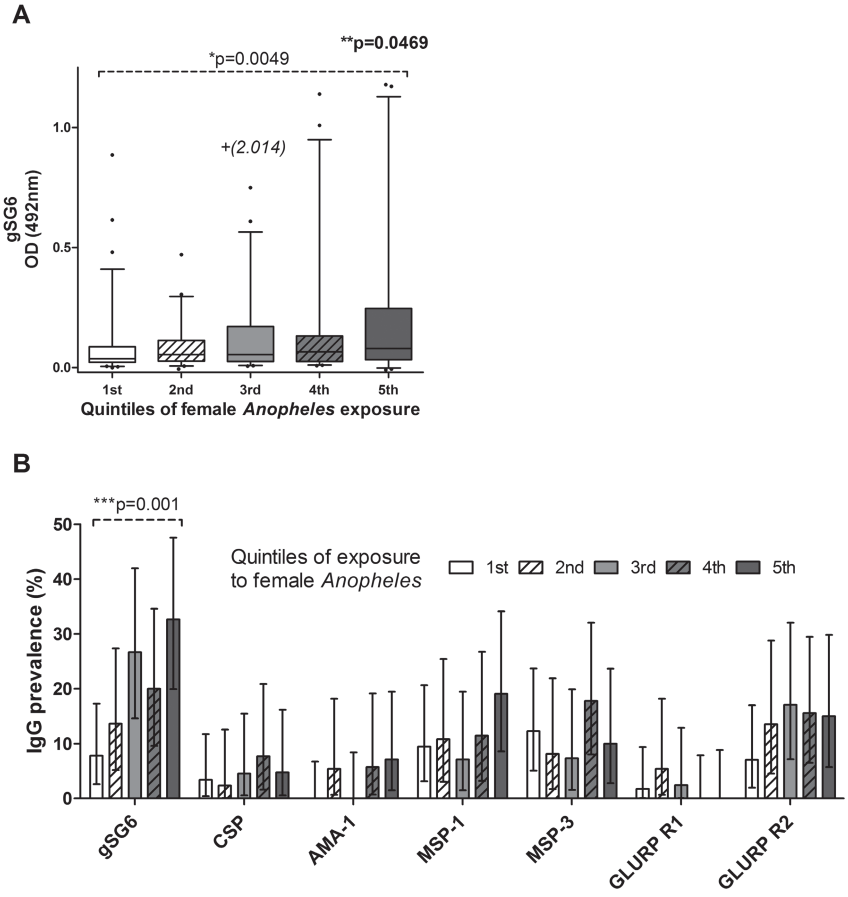

Figure 4. IgG responses to gSG6 and *P. falciparum* antigens, grouped into quintiles of household *Anopheles* exposure. A. Box plots showing anti-gSG6 IgG level between groups sorted according to *Anopheles* exposure in quintiles. Boxes show the median and 25th/75th percentiles, whiskers show the 5th/95th percentiles, and outliers are represented by dots. Where outliers were excluded from the graph but not analysis they are marked with a + and included in parentheses. P values for pairwise comparisons were determined by Mann-Whitney test with Bonferroni correction (*), and for all groups by Kruskal-Wallis test (**). **B.** Seroprevalence of anti-gSG6 and anti-*P. falciparum* IgG antibodies plotted against *Anopheles* exposure in quintiles. Error bars indicate 95% confidence intervals (CI). P values were determined by a test for trend in proportions (***).

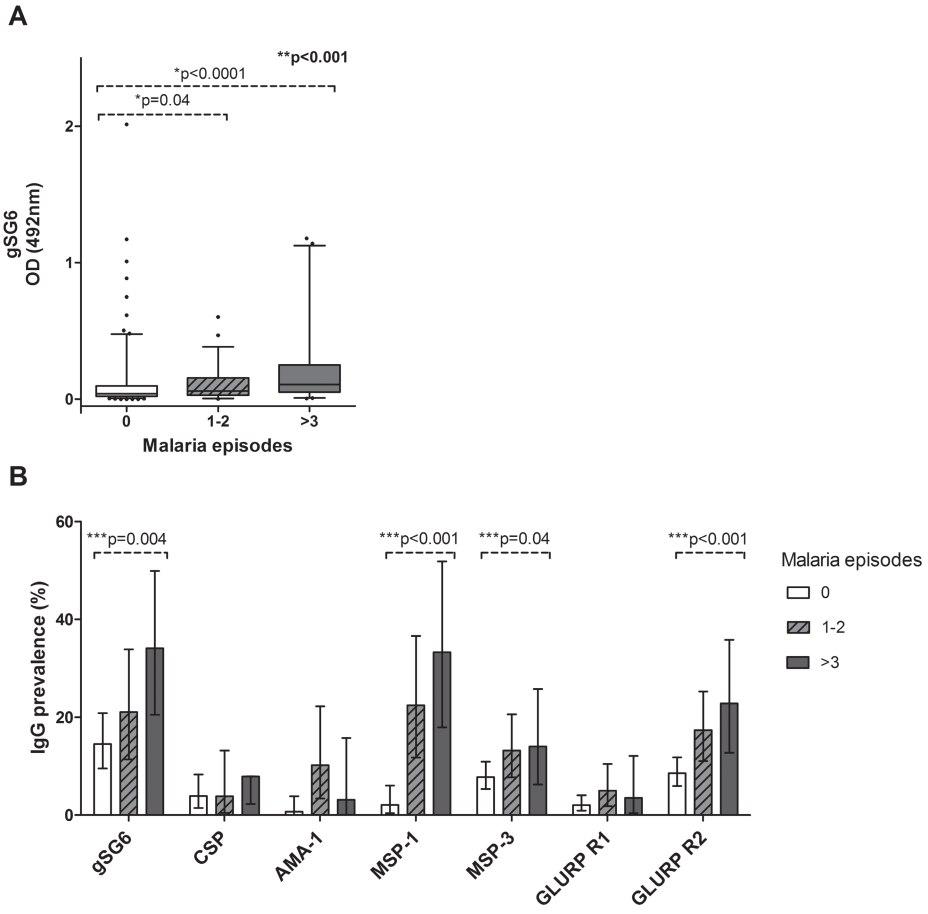

Figure 5. IgG responses to gSG6 and *P. falciparum* antigens, plotted against malaria incidence after serum collection. Malaria incidence is grouped into 0 episodes, 1-2 episodes or >3 episodes. **A.** Box plots showing anti-gSG6 IgG level between groups sorted according to malaria incidence subsequent to serological sampling. Boxes, whiskers and P values are as in *figure 4*. n=269. **B.** Seroprevalence of anti-gSG6 and anti-*P. falciparum* IgG antibodies plotted against grouped malaria incidence. Sample sizes vary by antigen according to the available serological methodology; CSP n=246, AMA-1 n=227, MSP-1 n=227, MSP-3 n=566, GLURP R1 n=566, GLURP R2 n=566. P values were determined by a test for trend in proportions (***). Error bars denote 95% CI.

Discussion

In the present study we show that the antibody responses of young children to the recombinant *A. gambiae* salivary protein, gSG6, reflect small scale spatial variation in malaria transmission, and are strongly associated with malaria risk in an area of moderate transmission intensity in northern Tanzania where *A. gambiae* and *An. funestus* are the main malaria vectors.

Reactivity to both the peptide and recombinant forms of the anopheline gSG6 protein has previously been associated with seasonal or regional patterns in mosquito exposure [45-48,55]. The current study is the first to describe antibody responses to the recombinant gSG6 protein in relation to village of residence, and individual level mosquito exposure and malaria incidence. For this, we utilised a detailed entomological dataset from Korogwe district, Tanzania, that revealed significant heterogeneity in *Anopheles* abundance between and within villages [11]. Despite generally low reactivity among our infant study population, anti-gSG6 IgG level and prevalence effectively described varying levels of exposure to *Anopheles* between subvillages, corroborating recent findings from Senegal where gSG6-P1 responses reflected spatial variation in *Anopheles* exposure between districts in urban Dakar [47]. The first studies to assess IgG responses to recombinant gSG6 were carried out in two rural villages in Burkina Faso, and revealed >50% seroprevalence in children during the peak transmission season [48]. The lower responses observed in this study confirm the lower transmission intensity in the current study area.

At the level of subvillages, anti-gSG6 antibody responses closely followed patterns in malaria incidence and community-level antibody responses to malaria-specific antigens AMA-1 and MSP-1_{19} [11]. This broad agreement in estimates of malaria incidence and *Anopheles* and malaria-specific antibody responses at subvillage level is unsurprising [45,48,49,55]. Patterns may diverge when assessed at an individual level, as *Anopheles* abundance and biting behaviour may be unevenly distributed between households [12,21,56] and intense mosquito exposure may not necessarily mean a high malaria exposure if anophelines are not infected. This commonly happens at the start of the wet season when mosquitoes have just emerged and are unlikely to have completed a sporogonic cycle [57], but mosquito sporozoite rates may also show spatial variation [11]. Associations between mosquito exposure, malaria incidence and immune responses are further complicated by the fact that individuals with the highest malaria exposure will acquire protective immunity most rapidly and may experience lower malaria incidence in some settings [58,59]. In general, it is complex to disentangle markers of exposure from markers of protection when analysing malaria blood stage antigens. Recent studies highlight the importance of considering malaria heterogeneity when determining the protective effect of antibody responses on clinical malaria. Initially, counterintuitive observations that higher blood stage immune responses were associated with increased malaria incidence [60,61], were explained by adjusting for heterogeneity in

malaria exposure and excluding non-parasitaemic individuals. This revealed a protective effect among immune responders, reflecting either true or surrogate humoral immune mediation [60]. This methodological challenge, first described by Bejon and colleagues [62,63], has highlighted the need for markers that capture heterogeneity in malaria exposure but are not associated with clinical protection [58,60,61]. Markers of mosquito exposure, as described in this manuscript, may play this role by identifying those individuals most at risk of malaria.

No clear associations were apparent between *Anopheles* exposure at an individual level and antibody responses to any of the malaria-specific antigens (CSP, AMA-1, MSP-1, MSP-3, GLURP R1, GLURP R2). Anti-CSP reactivity might be expected to correlate with exposure to infected mosquito bites and therefore perhaps also with overall mosquito biting, but in our analysis did not. This may be a consequence of the relatively small sample size, and low EIR [11,21]; in moderate to low endemic areas the proportion of infected vectors is frequently lower than 1% [15,64,65]. Contrary to this, individual-level anti-gSG6 responses were strongly associated with household *Anopheles* exposure. Interestingly, mosquito exposure was assessed towards the end of the study, starting approximately 15 months after the serum sample that was used for serology was collected. This suggests that heterogeneity in mosquito exposure is consistent over time in our study area, supporting the hypothesis of stable hotspots of malaria transmission [10,22].

We previously showed that antibody responses to blood stage malaria antigens determined in clinic attendees reliably predicted spatial patterns in malaria incidence in a cohort of children living in the same area [11]. We here extended these analyses and showed that an individual's antibody responses to MSP-1, MSP-3 and GLURP-R2 are all positively associated with subsequent malaria incidence. The selection of malaria antigens we used in this study was not intended to be exhaustive, nor did we aim to identify the malaria antigen with the highest discriminative power to detect variation in malaria exposure. We chose 4 malaria antigens to put our findings with gSG6 in an epidemiological context. Our findings are consistent with previous reports from areas of heterogeneous exposure where malaria specific antibody responses as markers of past exposure predict future exposure [60,61]. Strikingly, in our analyses anti-gSG6 responses also provided a strong association with malaria incidence, indicating that malaria heterogeneity is associated with heterogeneous biting behaviour [12]. Unlike responses to transmission and blood stage malaria antigens [65,66] responses to gSG6 confer no protection to malaria, thus avoiding any confounding associations with immunity and malaria incidence. In such a way, the gSG6 assay may provide a useful marker for exposure to malaria for use in clinical studies [58].

Though the sampling framework of the current study was not designed to evaluate the temporal dynamics of the anti-gSG6 response, there are indications that, as with responses to the salivary proteins of other haematophagous arthropods, it elicits short lived antibody responses, reflecting only recent *Anopheles* exposure [45,46,48]. As blood-feeding is

transitory, and saliva is only released into the skin during probing with the majority likely to be re-ingested with the blood meal, this limits the development of a humoral immune response to mosquito saliva [67-69]. This short exposure to antigen explains the low anti-gSG6 responses observed among children from Korogwe. These low level responses highlight inherent problems in assessing exposure using an arbitrarily defined cut off for seropositive individuals. Identifying individuals never exposed to malaria is relatively straightforward but the same cannot be said for individuals never exposed to *Anopheles*, a genus which has a very wide geographical distribution. The nature of mosquito feeding, with the strength of the correlations observed in our analyses between spatial and individual level mosquito exposure and antibody OD, supports the use of antibody level rather than seroprevalence as a finer tool for assessment of *Anopheles* exposure intensity.

Conclusions

This is the first report that antibody responses to the recombinant *A. gambiae* salivary protein gSG6 in children can reflect small-scale spatial variation in exposure to anophelines at village and household level. Importantly, our analysis also provides the first evidence for a reliable association between malaria incidence and anti-gSG6 response; a relationship only previously observed using whole *A. gambiae* saliva [44]. Caution is required in extrapolating findings from this study to other age groups because our analyses were restricted to plasma samples from children aged 9 months and a role of maternal transfer of IgG during breastfeeding can therefore not be excluded. This limitation of the current study does not alter our conclusions that these antibody responses are suitable markers of micro-epidemiological differences in *Anopheles* exposure. Potential uses for this assay include establishing *Anopheles* biting exposure to include indoor and outdoor biting, controlling for exposure in highly heterogeneous settings, and as a measure of receptivity to inform programs that are moving toward elimination where there is a high risk for re-introduction. However, its utility in low endemic and pre-elimination settings first needs to be assessed [8]. To this end, it will be important to establish the assays suitability for use with scalable antibody sources such as dried filter paper blood-spots. The identification and analysis of other salivary proteins may also help increase the sensitivity of the approach in such settings [70].

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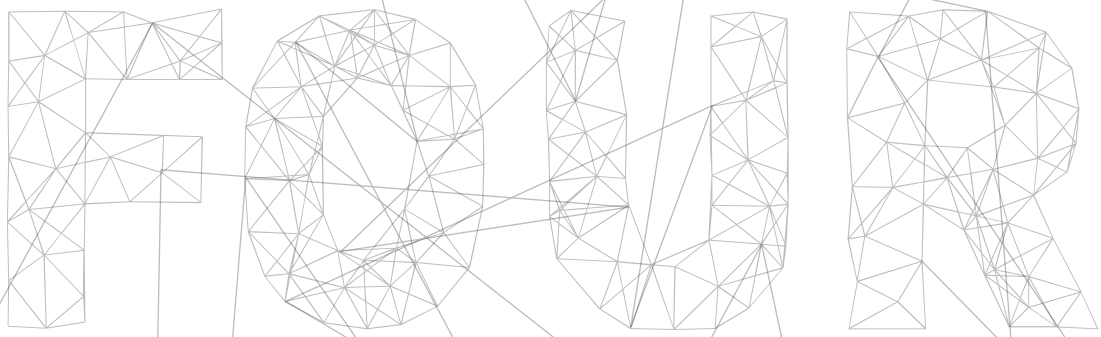
FOUR

Chapter 4

Assessing the infectious reservoir of falciparum malaria: past and future

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Trends in Parasitology. 2015;31(7):287-96

Abstract

Renewed interest in malaria eradication has placed greater emphasis on the development of tools to interrupt *Plasmodium* transmission, such as transmission-blocking vaccines. However, effective deployment of such tools is likely to depend on improving our understanding of which individuals transmit infections to mosquitoes. To date, only a handful of studies have directly determined the infectiousness of individuals in endemic populations. Here we review these studies and their relative merits. We also highlight factors influencing transmission potential that are not normally considered: the duration of human infectiousness, frequency of sampling by mosquitoes, and variation in vector competence among different mosquito populations. We argue that more comprehensive xenodiagnostic assessments of infectivity are necessary to accurately quantify the infectious reservoir and better target interventions.

Glossary

Endophilic: arthropod behaviour describing a preference for resting indoors after blood feeding.

Exophagic: arthropod behaviour describing a preference for blood feeding outdoors.

Exophilic: arthropod behaviour describing a preference for resting outdoors after blood feeding.

Gametocyte: the sexual stages of the malaria parasite capable of reproduction in the mosquito. Female and male gametocytes circulate in the human peripheral blood, where they may be ingested by blood-feeding *Anopheles* mosquitoes and begin sexual development.

Mosquito feeding assay: xenodiagnostic assays developed to determine the infectiousness of *Plasmodium* gametocytes to *Anopheles* mosquitoes. Mosquito feeding assay may refer to skin feeding assays, in which mosquitoes are allowed to feed directly on a subject's skin, to direct membrane feeding assays, in which mosquitoes feed on venous blood from a subject maintained at body temperature in a membrane feeding device, or to standard membrane feeding assays, in which mosquitoes feed on cultured gametocytes in a membrane-based feeder system.

Perennial transmission: malaria transmission may be described as perennial where environmental and climatic conditions allow parasite transmission throughout the year.

Seasonal transmission: malaria transmission may be described as seasonal where mosquito biting and thus transmission are clustered temporally, generally occurring following predictable periods of rain and subsequently decreasing in periods of drought.

Transmission-blocking immunity: the development of humoral immunity to antigens present during gametocyte development. Antibodies specific to gametocyte antigens may be ingested along with mature gametocytes during blood feeding. If these antibodies target proteins essential for parasite development (e.g., Pfs48/45, Pfs230) and are sufficiently abundant, antibody interaction can prevent parasite development and transmission potential is reduced or entirely blocked. There is a growing body of evidence for the development of transmission-blocking immunity in individuals naturally exposed to malaria infection.

Xenodiagnosis: in this context, is a method to determine the infectiousness of a potentially parasitized host (i.e., malaria-infected humans) by allowing vectors (i.e., mosquitoes) to feed on the subject. Infectiousness is assessed by the infection status of the vector (i.e., mosquito) after a suitable period of parasite development.

Reducing transmission of malaria: the future of elimination programs

The burden of malaria has declined in many endemic settings in Africa and elsewhere [1]. Local malaria elimination is considered achievable with current control approaches in some of these areas when transmission intensity is low and reintroduction unlikely [2, 3]. However, in most endemic areas operational and technical limitations are likely to hinder the complete interruption of transmission [4, 5]. New or renewed tools aimed specifically at interrupting the spread of *Plasmodium* species from human to mosquito may therefore be critical for future elimination programs [6, 7], especially if transmission efficiency increases as parasite prevalence goes down [8]. Un-like traditional control strategies, which aim to reduce severe morbidity in vulnerable populations, the effective-ness of transmission-reducing interventions (TRIs) hinges on their coverage of individuals responsible for transmission to mosquitoes regardless of their symptomatic status [9]. Despite the central importance of human infectivity for TRI [10], there have been few direct assessments of this. Here we discuss previous studies that aimed to directly assess infectivity at the population level, examine factors necessary to link these controlled transmission experiments with transmission in nature, and advocate the next steps that will provide the key information necessary to better target the human infectious reservoir for malaria.

Quantifying the human infectious reservoir of malaria (1957–2014)

In infected humans, the life cycle of a small portion of the total malaria parasite population culminates the differentiation and maturation into gametocytes (see Glossary). When an anopheline mosquito feeds on blood containing mature gametocytes some may be ingested, which then fuse and undergo sporogonic development making the insect infectious to humans. The human component of the infectious reservoir is the proportion of a population that is capable of infecting mosquitoes [11].

In community surveys, data from microscopy typically show that children and infants harbour *Plasmodium* gametocytes more commonly and in greater numbers than older age groups [12]. Such observations have long galvanized belief among the scientific community that young individuals represent the main source of infection for mosquitoes [12]. However, in the late 1940s and early 1950s xenodiagnostic assessments revealed that the presence of gametocytes in blood films was not a prerequisite for onward transmission [12–15]. The first population-based assessment of human infectivity to mosquitoes was conducted in rural Liberia, where individuals living in an endemic region were recruited for mosquito feeding experiments regardless of their parasite status [16]. While this study found that young children were much more infectious to mosquitoes, it also showed that, when estimates were adjusted for the demographic composition of the population, all age groups were relevant contributors to malaria transmission (**Box 1**). In nearly 60 years since this study, a handful of similar surveys have been reported (**Tables 1** and **2**). In general, these studies

Table 1. Summary of studies assessing the malaria infectious reservoir where recruitment was conducted without regard for parasite status [a]

Study location	Local transmission setting	Feeding method	Assay endpoint[b]	Species	Prevalence of infectiousness (%)[c]	Proportional contribution to reservoir (%)[d]				Mosquito infection probability[e]	Reference
						1 - 4	5 - 9	10 - 19	≥20		
Papua New Guinea	Moderate perennial	Direct & Membrane	Oocysts	*An. farauti*	3.8	22.7	32.9	30.3	14.2	0.013	(19)
						1 - 4	5 - 14	≥15			
Liberia	Intense perennial	Direct	Oocysts	*A. gambiae*	10.6	40.5	28.4	31.1		0.023	(16)
Kenya	Intense perennial	Direct	Oocysts & ELISA	*A. gambiae*	10.1	23.2	36.1	40.7		0.010	(17)
Burkina Faso	Intense seasonal	Membrane	Oocysts	*A. gambiae*	48.0	ND[f]	39.7	60.3		0.055	(76)
Cameroon (Bondi)	Intense seasonal	Direct	Oocysts	*A. gambiae*	7.4	28.8	49.2	21.9		0.024	(18)
Cameroon (Mengang)	Intense seasonal	Direct	Oocysts	*A. gambiae*	8.2	34.7	33.9	31.4		0.011	(18)
Senegal	Low, unstable	Direct	Oocysts	An. arabiensis	8.7	ND[f]	51.8	48.2		0.002	(31)

[a] Only studies that have utilized demographic and mosquito feeding data to characterize the human infectious reservoir of *Plasmodium* in endemic populations are included. All studies were conducted in areas where *Plasmodium falciparum* and non-*falciparum* species cocirculate, so the outcome of feeding assays cannot be attributed unambiguously to *falciparum* malaria. One additional study not included in the table utilized population and xenodiagnostic data to quantify the malaria infectious reservoir in Sri Lanka [79]. This study recruited individuals with incident malaria over a 17-month period, most of whom had *Plasmodium vivax*. The study design and analysis was suitably different as to exclude the possibility of direct comparison with the other studies presented in the table.

[b] Assay end point was either: (i) oocysts counted on the mosquito midgut by microscopy; or (ii) oocysts counted by microscopy or positivity of remaining live mosquitoes at the end of the experiment (day 15 post-infection) in circumsporozoite protein (CSP) ELISA [17].

[c] Prevalence of infectiousness was calculated as the combined prevalence of individuals infectious to ≥1 mosquito in each age category, adjusted for the proportion of the total population constituted by the age groups.

[d] Proportional contribution to the reservoir presents the proportional contributions of three or four age groups to the population-adjusted reservoir, with age groupings combined where the data in the original publication allowed.

[e] Mosquito infection probability corresponds to the probability that a mosquito biting a random individual in a population will develop infection. This measure is presented as in the published data or was calculated by combining demographic data, age-specific prevalence of infectiousness, and age-specific mosquito infection rates during infective membrane feeding experiments.

[f] ND, study unable to perform mosquito feeds on individuals <5 years of age. Data have been adjusted to represent the infectious reservoir of individuals >5 years only.

show that while young children are consistently more likely to be infectious per se [17, 18], the contribution of older age groups is by no means negligible; in previous xenodiagnostic surveys that were able to sample individuals of all ages, individuals >15 years old constituted between 21.9% and 40.7% of the total infectious reservoir [16–19]. Several studies have assessed human–mosquito transmission using entomological parameters [20]. However, these studies provide only broad population estimates of infectiousness and they are neither able to describe the distribution of the reservoir among different members of the population nor allow an examination of the role of parasite density and infection dynamics.

Box 1. Demography and the malaria infectious reservoir

In previous surveys, the 'bottom-heavy' age structure typical of malaria endemic regions has acted to boost the contribution of more infectious children to the total malaria infectious reservoir, while less infectious adults, representing approximately half of most populations, generally contributed to a lesser extent. Recent demographic estimations show that Sub-Saharan African populations are still disproportionately young: approximately 17% and 27% of the population are aged 0–4 and 5–14 years, respectively (http:// esa.un.org/wpp/). The influence of demography on patterns of transmission needs to be considered in light of the numerous other factors affecting the likelihood of infectious individuals contributing to mosquito infections. Although infants and children are over-represented in endemic areas they may be the age group least available and, perhaps, least attractive to mosquitoes (see 'Determinants of human transmission potential: the need for data'). Conversely, older children and adolescents may represent a privileged group for human–mosquito transmission; individuals in this age group possess moderate gametocyte densities (approximately double the infectivity of individuals >15 years), lack the severe symptoms that prompt treatment, and appear comparatively vulnerable to mosquito biting (see **Figure 1** in main text). Women in pregnancy have been observed to attract more than twice as many anophelines as non-pregnant women over short distances [21]. However, such observations must be weighed against behavioural and demographic parameters unique to this group. Census and fertility rate data show that pregnant women comprise a small proportion of African populations. In Zambia, 8.7% of women of child-bearing age were pregnant at any one time in 2013, which equates to 2–2.5% of the entire population (http://www. dhsprogram.com/pubs/pdf/FR304/FR304.pdf). Pregnant women are also preferentially targeted by net and drug treatment campaigns, so the high attractiveness to mosquitoes of this small proportion of the population may be balanced by interventions.

Ultimately, determining the infectiousness of different age groups relative to their representation in the population is just one necessary step in characterizing the infectious reservoir. Contribution to transmission is influenced not only by intrinsic parasite and human determinants of infectivity, reflected in the outcome of mosquito feeding assays, but also by various additional factors that make the capacity to infect mosquitoes in controlled experiments distinct from the probability of onward transmission in nature (e.g., human exposure to mosquito biting) (**Figure 1**). These factors are not commonly taken into account and are not uniform across time or space or between individuals of different ages. To more accurately reflect transmission in its epidemiological context, these elements need to be considered and incorporated into future assessments.

Table 2. Summary of studies assessing the malaria infectious reservoir that recruited only gametocyte-positive individuals for feeding assays [a]

Study location	Local transmission setting	Feeding method	Assay endpoint[b]	Species	Prevalence of infectiousness (%)[c]	Proportional contribution to reservoir (%)[d]				Mosquito infection probability[e]	Reference
						1 - 4	5 - 9	10 - 19	≥20		
The Gambia	Intense seasonal	Membrane	Oocysts	*A. gambiae*	5.6	17.5	21.7	22.2	37.9	0.015	(11)
Tanzania	Intense perennial	Membrane	Oocysts	*A. gambiae s.l.*	3.2	28.4	23.1	14.2	34.0	0.007	(11)
Papua New Guinea[f]	Moderate perennial	Direct & Membrane	Oocysts	*An. farauti*	3.6 (1.4)	48.0	12.4	24.0	15.5	0.012 (0.004)	(19)

[a] Only studies that have utilized demographic and mosquito feeding data to characterize the human infectious reservoir of *Plasmodium* in endemic populations are included. All studies were conducted in areas where *Plasmodium falciparum* and non-*falciparum* species cocirculate, so the outcome of feeding assays cannot be attributed unambiguously to *falciparum* malaria.

[b] Assay end point was oocysts counted on the mosquito midgut by microscopy.

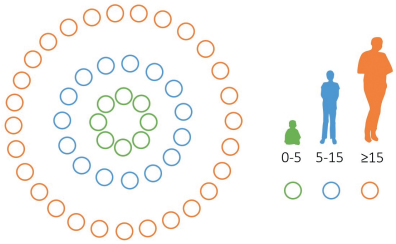
[c] Prevalence of infectiousness was calculated as the combined prevalence of individuals infectious to ≥1 mosquito in each age category, adjusted for the proportion of the total population constituted by the age groups.

[d] Proportional contribution to the reservoir represents the proportional contributions of three or four age groups to the population-adjusted reservoir, with age groupings combined where the data in the original publication allowed.

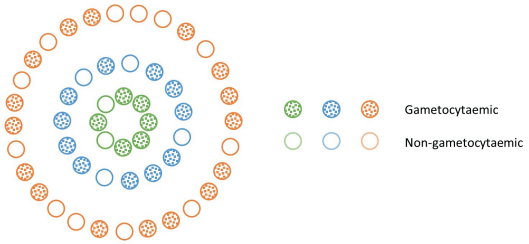
[e] Mosquito infection probability corresponds to the probability that a mosquito biting a random individual in a population will develop infection. This measure is presented as in the published data or was calculated by combining demographic data, age-specific prevalence of infectiousness, and age-specific mosquito infection rates during infective membrane feeding experiments.

[f] For this study, all species and *P. falciparum*-specific (within parenthesis) estimates are presented.

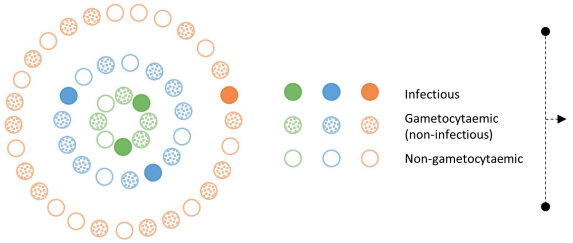
A. Age-stratified population



B. Proportion of individuals with gametocytes



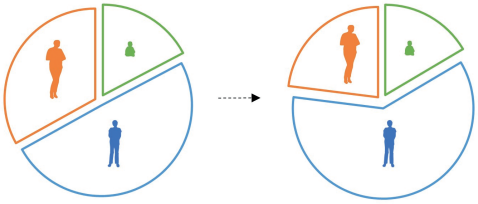
C. Proportion of individuals infective to mosquitoes



Proportional contribution to total infectious reservoir by age

1. Contribution based solely on the prevalence of infectious individuals

D. Individuals adjusted for surface area/mosquito biting frequency



2. Contribution adjusted for body size

3. Contribution adjusted for body size *and* relative exposure

Figure 1. Age contributions to the infectious reservoir. (**A**) Individuals in the figure are represented by circles in three age groups: <5, 5–15, and >15 years. The abundance of individuals in each group reflects a simplified population age structure in Sub-Saharan Africa as described in Box 1 and in the studies detailed in Tables 1 and 2 (<5 = 15%, 5–15 = 30%, >15 = 55%) (http://esa.un.org/wpp/). (**B**) Speckling within circles represents the presence of *Plasmodium falciparum* gametocytes detectable using quantitative nucleic acid sequence-based amplification (QT-NASBA); age-specific prevalences presented here were taken from an area of intense seasonal transmission [87]. (**C**) Solid filled circles represent who might infect mosquitoes in a mosquito feeding assay. Some (or most) gametocytemic individuals may not be infectious for various reasons, including gametocyte density, maturity, recent treatment, and transmission-blocking immunity. Infectiousness prevalence for the three age groups (<5 = 25%, 5–15 = 13%, >15 = 7%) is based on surveys described in Table 1 [16–18]. Pie Chart 1 demonstrates the proportional contribution of each age group to the total population infectious reservoir. Demographic adjustment is unnecessary as this example directly represents the age composition of a typical population. Of the theoretical total infectious population [9.8% (5/51 individuals)], individuals in the age groups <5, 5–15, and >15 years account for 40%, 40%, and 20% of the reservoir, respectively. (**D**) Equally exposed individuals of different ages are differently attractive to mosquito biting, partly due to differences in body surface area. To reflect this, individual circles are given a surface area proportional to the average body surface area of individuals in the three age groups (<5 = 0.4 m2, 5–15 = 1.2 m^2, >15 = 1.6 m^2; ratio of surface area 1:3:4) using data from Port et al. showing that body surface area correlated positively with mosquito biting rate [45]). Pie Chart 2 illustrates the potential effect of increased mosquito sampling due to body surface area in each age group on the infectious reservoir. Finally, to account for some of the age-related differences in exposure intensity, the percentage bed net use recorded in individuals from Western Kenya (coverage: <5 = 42%, 6–17 = 26%, >17 = 57.7%; ratio of exposure 1:1.2:0.7) was used to further adjust the surface area weighted contributions to the infectious reservoir (pie Chart 3) [88].

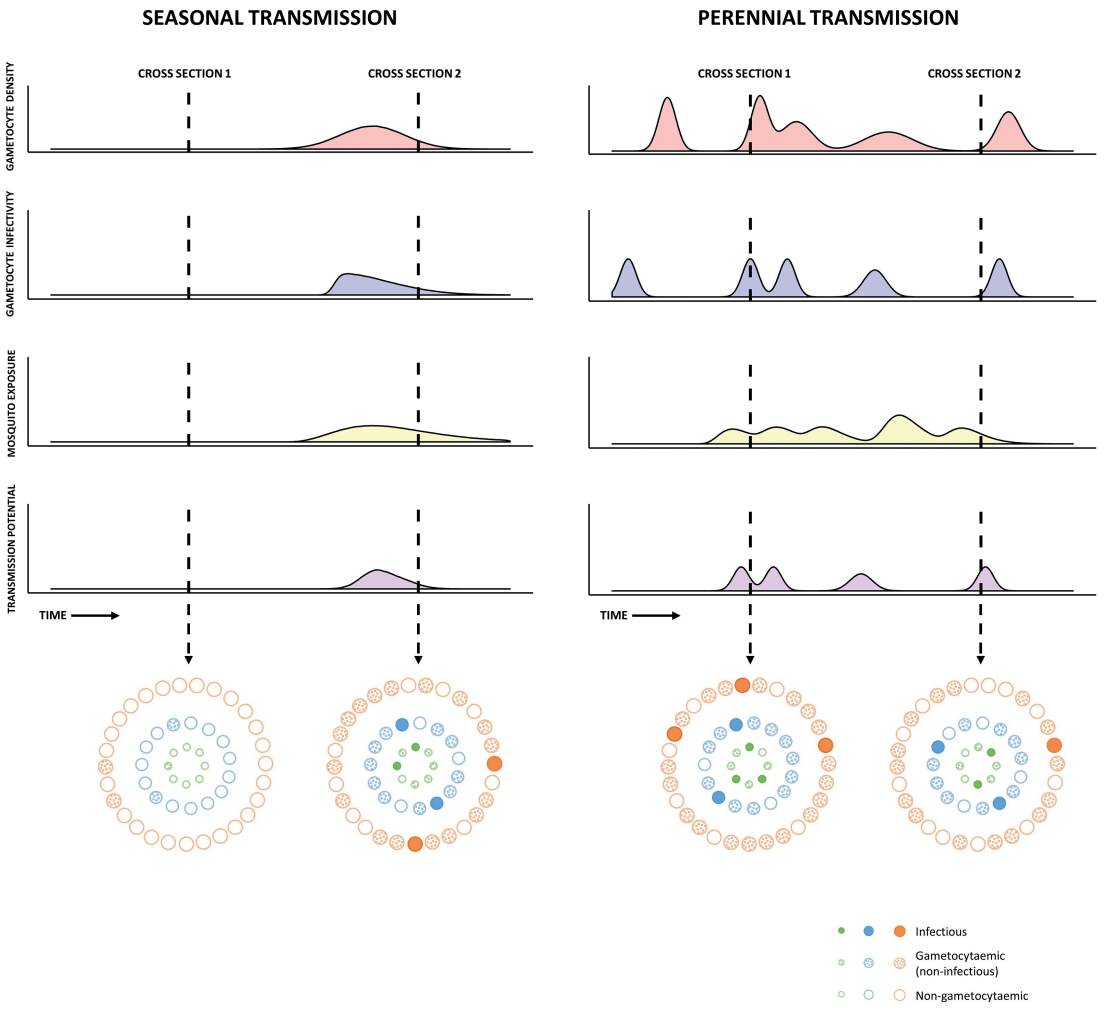

SEASONAL TRANSMISSION
PERENNIAL TRANSMISSION
CROSS SECTION 1
CROSS SECTION 2
GAMETOCYTE DENSITY
GAMETOCYTE INFECTIVITY
MOSQUITO EXPOSURE
TRANSMISSION POTENTIAL
TIME
Infectious
Gametocytaemic (non-infectious)
Non-gametocytaemic

Figure 2. Cross-sectional surveys and infectious reservoir dynamics. Data represent hypothetical annual transmission measures in two different endemic settings of seasonal and perennial malaria transmission. The broken line corresponds to the timing of cross-sectional xenodiagnostic surveys. The population plots underneath the main charts reflect hypothetical population-level infectiousness, as would be measured in direct assessments of the reservoir. Individuals in the plots are represented by circles in three age groups: <5, 5–15, and >15 years. The abundance of individuals in each group reflects a simplified population age structure in Sub-Saharan Africa, as described in Box 1 and in the studies detailed in Tables 1 and 2 (<5 = 15%, 5–15 = 30%, >15 = 55%) (http://esa.un.org/wpp/). Speckling within circles represents the presence of *Plasmodium falciparum* gametocytes; age-specific prevalence is hypothetical. Solid filled circles represent who might infect mosquitoes in a mosquito feeding assay; infectiousness prevalence for the three age groups is hypothetical. To reflect differences in body size and potentially attractiveness to mosquitoes, individual circles are given a surface area proportional to the average body surface area of individuals in the three age groups (<5 = 0.4 m^2, 5–15 = 1.2 m^2, >15 = 1.6 m^2; ratio of surface area 1:3:4) using data from Port et al. showing that body surface area correlated positively with mosquito biting rate [45]. The combined surface area of individuals in the plots can be interpreted broadly as the contribution each group makes to the total infectious reservoir. Combined, the figure illustrates that the total infectious reservoir and the proportional contribution of differently aged individuals are likely to vary, potentially significantly, over time in response to the dynamicity of numerous factors. Although variations in transmission potential would probably be clearest between surveys where transmission is seasonal, age-specific contributions may vary with time in areas where transmission is intense and perennial.

Determinants of human transmission potential: the need for data

Duration and dynamics of infectivity

The likelihood of transmission to *Anopheles* mosquitoes is determined primarily by gametocyte density, fitness, and circulation time. The nonlinear relationship between gametocyte density and mosquito infection risk has been described several times [21–23]. Yet, because of the relatively high proportion of low-density parasitemias and gametocytemias in natural infections, there is increasing focus on how important these often subpatent infections are for the maintenance of transmission [24, 25]. While the limited sensitivity of microscopy to detect gametocytes is well established [21], many claims of submicroscopic gametocyte densities contributing to transmission are based on microscopy-positive asexual parasite carriers with ac-companying submicroscopic gametocyte densities. This is an important difference when considering how current diagnostics may capture the human infectious reservoir [26, 27]; infections with submicroscopic gametocytes that accompany patent asexual parasites are detectable by conventional diagnostics while infections with no microscopically detectable parasite stages require more sensitive diagnostics [28].

Because of their cross-sectional nature, previous xenodiagnostic surveys have not formally examined the duration of infectiousness, another key factor influencing transmission potential. Data from malaria therapy studies suggest that the infectiousness of an individual infection can last for many months [29]. However, it is difficult to extrapolate the observations from a limited age range of malaria-naive individuals to endemic populations. Broadly, in endemic settings children (possessing limited immunity) are likely to have more acute, higher-density infections that often require treatment [30]. This may result in cross-sectional surveys missing recently treated infections and identifying only a limited number of highly infectious children without recent treatment. Conversely, adults and older children (often semi-immune, and presenting few clinical symptoms) would have longer, chronic infections, which could result in longer infectious periods, balanced by lower infectivity at individual time points [31]. These patterns of infection are likely to differ with transmission intensity. In areas of intense malaria transmission, children may have repeated malaria infections with relatively high gametocyte densities and a high likelihood of malaria transmission (**Figure 2**). In areas with lower transmission, including areas that have reduced transmission in the context of an elimination program, infection duration is likely to have a more significant influence on the infectious reservoir. At present only one study has directly assessed population-level infectiousness in an area of low malaria transmission [32]. Because of the utility of these data to control and elimination programs, further studies assessing the infectious reservoir in areas of low transmission would be advantageous.

In an individual infection, infectiousness per gametocyte may be higher at the beginning of an infection than at the end because of changes in parasite competency [33], sex ratio [34, 35], and, possibly, the development of sexual stage immune responses [36–38]. Transmission-blocking immunity is boosted by recent exposure to gametocytes [37] and

may reduce infectiousness during the course of infections that last several weeks or months. Superinfecting clones influence parasite growth dynamics, including total gametocyte biomass [39], and multiplicity of infection could in itself be a determinant of transmissibility [40]. This combination of factors might also indicate that the infectiousness of one infection may not reflect the infectiousness of subsequent infections (when multiplicity of infection and transmission-blocking immunity may differ), suggesting greater complexity than that estimated from a single time point during the transmission season.

Mosquito exposure

Artificial feeding experiments in xenodiagnostic surveys have tended to use fixed numbers of mosquitoes, improving the precision of estimates of infectiousness by maximizing mosquito sample sizes. However, in any endemic area mosquito biting rates of humans vary considerably. As an example, a recent study in Uganda found that biting rates varied from 270 to 7399 bites/year between study sites, with each site showing distinct seasonal fluctuations [41]. The transmission potential of infectious individuals will be determined by the rate at which they are sampled by local mosquitoes and the competence of these vectors. Where vector densities and survival rates are low, the number of mosquitoes that become infected and subsequently transmit their infections will be very low even if there are infectious individuals in the human population. Although essential to accurately characterize individual-specific contributions to transmission, these entomological parameters have never been measured in conjunction with xenodiagnostic surveys.

Attractiveness to mosquitoes

In the early 1950s, Muirhead-Thomson observed that Jamaican adults attracted more *Anopheles* mosquitoes than equally exposed children [42]. The conclusions of this study are supported by later reports in which wild *Anopheles* were allowed to feed on unprotected humans under observation [43, 44] or ABO blood typing allowed the identification of blood-meal origin [45], although some studies show more random biting selection [46]. Carnevale et al. near Brazzaville in the Republic of Congo described the biting behaviours of approximately 6500 mosquitoes on 24 individuals of all ages [43]. *Anopheles* biting increased with age, with individuals >20 years old being bitten three times more than <2-year-olds. Port et al. were able to ascribe this age-related biting pattern to increased body weight and surface area [45]. In addition to increased surface area, body temperature and chemical cues change with age and physiological maturity and may be associated with attractiveness [47–49]. This might also help explain why pregnant women are thought to be more attractive to mosquitoes than non-pregnant women [50]. Muirhead-Thomson also observed that infants reacted more actively in sleep to the probing of mosquitoes than adults and that mosquitoes were less likely to feed on infants even after alighting on their skin [42]. Data are less robust on the influence of *Plasmodium* infection on mosquito attractive-ness. Reports from animal

models and studies on a small number of humans infected with *P. falciparum* suggest that the presence of gametocytes may increase attractive-ness to anopheline mosquitoes [51, 52], possibly due to the production of attractive volatiles [53]. It is unclear whether the small biomass of gametocytes could have specific systemic effects that would increase the attractiveness of children (harbouring higher gametocyte densities) and whether this might counteract the reduced attraction associated with their smaller surface area.

Availability to mosquitoes

However attractive and infectious individuals are, they must be available to mosquitoes for feeding and subsequent transmission to occur. This availability is shaped primarily by human social and behavioural practices, including use of mosquito nets and sleeping patterns.

Age-specific net use depends on the setting and extent of ownership, but in areas with good coverage appears highest among young children (<5 years) and lowest among older children and adolescents (5–15 years) [54–57]. In addition to bed nets' community-wide effect on transmission, they may also reduce an individual's chance of infecting mosquitoes and divert vectors to other blood-meal sources. In The Gambia, the introduction of insecticide-treated nets (ITNs) protected children against malaria and diverted mosquito bites to other hosts (animals or adults) [58]. Net distribution has also been linked to changes in vector behaviour and species composition; *Anopheles gambiae* and *Anopheles funestus* have shown signs of responsive exophagy [59] and there are observations that peak biting in these species may shift to the early evening and morning [60, 61]. Net-related reductions in *A. gambiae s.s.* populations have also been linked to increasingly dominant roles for *A. funestus* [62] and *Anopheles arabiensis* [63].

These elements, together with sleeping times and time spent indoors or outdoors at night, determine availability to different mosquito populations. Children might be expected to sleep earlier than adults, but the protection afforded by long periods of time under bed nets depends on when local vectors are most active. Data on time spent indoors, time spent under nets, and the biting activity of local vectors can be combined to determine the true protective efficacy of bed nets by age [64]. This is an informative composite measure that could be used to estimate age-specific patterns of mosquito exposure.

Effectively delineating the interaction of mosquito exposure and subsequent malaria transmission is difficult. Individuals identified as infectious in xenodiagnostic surveys must have been exposed to malaria vectors at some stage, but heterogeneity in attractiveness and the degree of exposure should still significantly influence their relative contribution to the infectious reservoir. Surveys quantifying mosquito exposure and infectiousness in the same population could provide more realistic estimates of age-specific contributions to transmission.

Heterogeneous mosquito populations

Although several vector species might contribute to local transmission in endemic areas, most xenodiagnostic surveys have been performed using *A. gambiae s.s.* (Tables 1 and 2), most of which were colony reared. Evidence is limited on whether infectiousness to *A. gambiae* during membrane or skin feeding assays corresponds to infectivity to other vector species. Two major malaria vectors, *A. funestus* and *A. arabiensis*, are distributed throughout Sub-Saharan Africa, often coexisting with *A. gambiae s.s.* [65]. The highly anthropophilic biting preferences of *A. funestus* [66] and its high longevity [67] and sporozoite rate [62, 68] (higher than *A. gambiae* in some endemic areas) indicate that a non-negligible part of malaria transmission might be sustained by this vector where it is present. Conversely, *A. arabiensis* is considered more exophilic and exophagic; in Burkina Faso, *A. arabiensis* was as susceptible to infection as *A. gambiae s.s.* in feeding assays [69], while in Senegal lower infection rates were observed in membrane feeding assays with *A. arabiensis* compared with *A. gambiae s.s.* [70]. The recent description of cryptic malaria vectors [71, 72] and conflicting results on the differential susceptibility of *A. gambiae s.s.* and *Anopheles coluzzi* [69, 70] (previously the molecular forms S and M of *A. gambiae*, respectively [73]), further illustrate the need to quantify transmission efficiency against the background of diverse vector populations.

Considerations for future studies

To more rigorously assess the human infectious reservoir using direct xenodiagnostic surveys, several methodological issues require consideration. In this section, we suggest suitable inclusion criteria and methods for measuring or predicting individual-level mosquito exposure. Other important factors that are discussed include the type of feeding assay, measures of infectiousness, and general study design.

Inclusion criteria for direct assessments of the infectious reservoir

There is abundant evidence that microscopy is insufficiently sensitive to detect low densities of asexual parasites and gametocytes [15, 19, 74–78]. Studies including only individuals with patent gametocytes [11, 19, 79] are likely to have underestimated the proportion of populations capable of infecting mosquitoes, and biased measures of mosquito infection probability (Table 2). Since low-density gametocyte carriage is prevalent in many endemic areas [25] and may represent a major source of secondary mosquito infections [22, 80, 81], studies aiming to characterize the human infectious reservoir should not have gametocyte positivity by microscopy as an inclusion criterion.

Defining infectiousness at the population level

The simplest method for quantifying the infectious reservoir combines the prevalence of people in different age groups infecting at least one mosquito and the proportion of those groups in the total population [16, 77]. Alternatively, population-level infectiousness may

be presented as mosquito infection probability (K), the likelihood of a mosquito becoming infected after feeding on any member of the population, calculated as the age-adjusted mosquito infection rate. Although the former metric has value for efforts that aim to identify or target all individuals who contribute to onward malaria transmission, the latter (mosquito infection probability) makes estimates more comparable between studies where the number of mosquitoes used in feeding assays may differ widely [18, 19] and captures variations in infectiousness intensity in different age groups that may not be apparent when reporting only infectiousness prevalence.

Assay methodology

There are two types of mosquito infection assay that have been used in previous surveys: direct skin feeding and membrane feeding assays (Tables 1 and 2). Direct feeds better reflect natural infection and avoid some of the technical challenges that limit the wide-scale use of mem-brane feeding assays (e.g., adapting mosquito colonies to membrane feeding, preventing gametocyte activation be-tween bleeding and feeding, a potential effect of anticoagulants) [82, 83]. Although their use is currently hindered by ethical concerns that have precluded their use in repeated assessments of mosquito infection from young children [82], further direct comparisons of xenodiagnostic surveys using skin and membrane feeding experiments would be highly valuable to parameterize the extent to which the latter reflect the natural situation. Unless direct skin feeding is acceptable in all age groups and with sufficient mosquito numbers to allow precise estimates of infection, studies aiming to characterize the full infectious reservoir in endemic populations should do so using direct membrane feeding assays using locally relevant malaria vectors.

Whether feeding assays measure infection by detection of oocysts in the mosquito gut or sporozoites in the salivary glands (Tables 1 and 2) should make little difference to the comparability of future studies, as even in low-intensity infections (such as those occurring commonly in nature) there is a close correlation between oocyst prevalence and the prevalence of subsequent salivary gland infection [84]. Salivary gland dissections are technically even more demanding than midgut dissections, will result in higher mosquito mortality between the day of feeding and the day sporozoites are first detectable, and impose additional safety measures for insectaries that need to be equipped for holding mosquitoes that form a biohazard to insectary personnel. We therefore suggest that detection of *Plasmodium* oocysts is the most viable measure of mosquito infection.

Measuring mosquito exposure

As outlined above, the number of mosquito bites an individual receives is influenced by their physical characteristics and behaviour [42, 45]. There is also spatial heterogeneity in exposure, with individuals living in households with higher vector densities (e.g., houses nearer to breeding sites) receiving more bites and having greater risk of disease than those

in households where vector density is lower [85]. Infectious reservoir assessments should ideally use a direct approach to quantify mosquito biting rates, simultaneously accounting for the availability and attractiveness of human hosts to mosquitoes: collection of mosquitoes from households and identification of blood-meal sources by DNA fingerprinting would provide reliable estimates of true exposure to mosquito bites. A recent study in Peru used this approach to show that adults were more often bitten by *Aedes aegypti* mosquitoes compared with children living in the same houses [86]. We suggest that in areas where transmission is dominated by indoor-biting mosquitoes, the rate of secondary mosquito infections could be calculated by multiplying individual-specific mosquito biting rates (determined experimentally using the above methods) by infectiousness (the probability that a mosquito develops infection after feeding, determined with mosquito feeding assays). This measure would be informative as it accounts for all major factors influencing transmission potential. An obvious problem with this approach is that only endophilic mosquitoes are sampled and there are currently no standardized sampling strategies for outdoor-biting vectors.

Where direct assessments are operationally impossible, or made unreliable by vector behaviour, we suggest that daily mosquito biting rates could be estimated for each study subject by collating mosquito (e.g., abundance, estimated indoor and outdoor biting rate), demographic (e.g., age, body surface area and attractiveness, house-hold conditions, distance to breeding sites, number of people living in the same house/room) and behavioural (e.g., sleeping times, net use) data, so that a rate of secondary mosquito infections could be broadly estimated by combination with experimentally determined infectiousness. **Figure 1** is a simplified and hypothetical re-presentation of the potential influence of some of these factors on the contribution of different age groups to the infectious reservoir.

Longitudinal studies with mosquito exposure estimation

Longitudinal studies with frequent follow-up would be valuable not only to estimate the duration of infectiousness in different age groups but also to assess whether some individuals or groups of individuals are infectious through-out a transmission season. This approach would help determine whether transmissibility is associated with clusters of higher malaria infection incidence; that is, where mosquito-to-human transmission is high. The more complete interpretation of such assessments could be made with concurrent mosquito biting rates.

Concluding remarks

We acknowledge that there are several malaria-endemic settings in which progress toward elimination has been made in the absence of detailed local knowledge of the infectious reservoir. However, malaria remains a threat in many countries, particularly in Africa, and resurgence due to natural receptivity for transmission and drug and insecticide resistance means that a better understanding of who within a community infects mosquitoes can only benefit malaria control.

The complexity of factors influencing malaria transmission means that studies aiming to fully understand how this occurs are challenging. Previous xenodiagnostic surveys provided valuable insights into the epidemiology of malaria transmission, but these studies need to be extended to simultaneously evaluate additional key factors. Moreover, the longitudinal nature of infectiousness during natural malaria infections needs to be considered in future studies, as this will influence the contribution of different age groups to transmission.

While human and parasite factors, including the intensity and duration of gametocytemia, affect infectiousness at the individual level, entomological factors dictate the transmission potential of each individual and the infectious reservoir as whole. Therefore, mosquito exposure assessments need to be an integral part of xenodiagnostic surveys if we are to effectively target the reservoir of infection (Box 2).

Box 2. Outstanding questions

If and how does human population infectiousness to mosquitoes differ in areas of low malaria transmission?

Are traditionally low endemic settings different from those under epidemiological transition (i.e. settings moving from moderate to low transmission as a result of heightened control activities)?

What is the duration of infectiousness of individuals to mosquitoes?

Are certain people infectious for longer periods and, if so, what are their characteristics?

How do fluctuations in mosquito densities and feeding rates affect the transmission potential of infectious humans?

Acknowledgments

The authors acknowledge the Bill & Melinda Gates Foundation for funding (grant OPP1034789).

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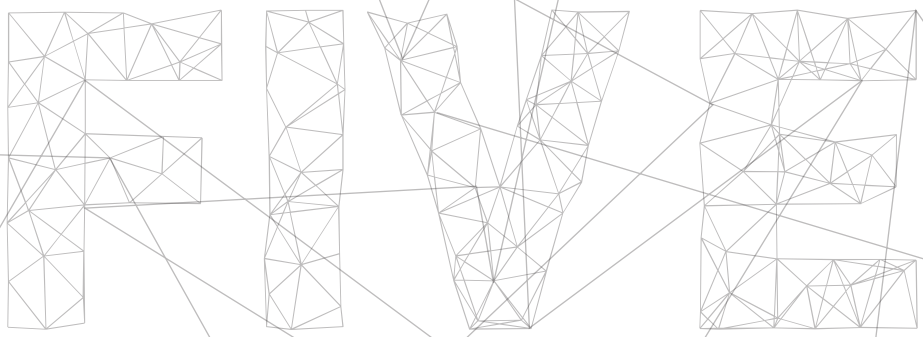
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Chapter 5a

A scalable assessment of *P. falciparum* transmission in the standard membrane-feeding assay, using transgenic parasites expressing Green fluorescent protein–Luciferase

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Journal of Infectious Diseases. 2014;210(9):1456-63

Abstract

Background

The development of drugs and vaccines to reduce malaria transmission is an important part of eradication plans. The transmission reducing activity (TRA) of these agents is currently determined in the standard membrane feeding assay (SMFA) based on subjective microscopical read-outs and with limitations in up-scaling and throughput.

Methods

Utilising a *Plasmodium falciparum* strain expressing the firefly luciferase protein, we present a luminescence based approach to SMFA evaluation that eliminates the requirement for mosquito dissections in favour of a simple approach where whole mosquitoes are homogenised and examined directly for luciferase activity.

Results

Analysis of 6860 *Anopheles stephensi* mosquitoes across 68 experimental feeds shows that the luminescence assay was as sensitive as microscopy for infection detection. The mean luminescence intensity of individual and pooled mosquitoes accurately quantifies mean oocyst intensity and generates comparable TRA estimates. The luminescence assay presented here could increase SMFA throughput so that 10-30 experimental feeds could be evaluated in a single 96-well plate.

Conclusions

This new method of assessing *Plasmodium* infection and transmission intensity could expedite the screening of novel drug compounds, vaccine candidates and sera from malaria exposed individuals for TRA. Luminescence-based estimates of oocyst intensity in individual mosquitoes should be interpreted with caution.

Introduction

With an estimated 627,000 deaths and approximately 207 million clinical cases annually, *Plasmodium falciparum* malaria remains the world's most devastating parasitic disease [1]. In recent years, large-scale distribution of highly effective artemisinin-based combination therapies and long-lasting insecticide-treated nets have contributed to a reduction in the burden of malaria in many endemic settings. Despite these positive trends, in the majority of areas where malaria is currently endemic, malaria elimination is unlikely to be achieved using only the available tools [2]. The development of drugs and vaccines with transmission reducing activity (TRA) has therefore become central to plans to eradicate the disease [3, 4].

The efficacy of agents with putative effects on *Plasmodium* transmission is determined under laboratory conditions, using the biological standard membrane feeding assay (SMFA). Mosquitoes are fed on blood containing cultured *Plasmodium* gametocytes, and the effect of components added to this surrogate blood-meal is determined around a week later by assessing the prevalence and intensity of established parasites in a sample of mosquitoes. Infection status is determined by the observation and quantification of *Plasmodium* oocysts on the dissected mosquito midgut. The TRA is defined as the percentage reduction in mean oocyst intensity between test and control mosquitoes [5, 6].

Although the SMFA is a powerful bio-assay, the technique's reliance on manual mosquito dissection and screening of individual mosquito midguts by microscopy makes its evaluation slow and laborious, and its interpretation inherently subjective [7, 8]. Recent studies have quantified the impact of the number of mosquito observations on the precision and variance of TRA estimates [5, 9], further highlighting the need for SMFA improvements that might facilitate the efficient and accurate evaluation of large numbers of mosquitoes. The use of green fluorescent protein (GFP)-expressing *Plasmodium berghei* has been reported as a first attempt to make the SMFA more scalable using a semiautomated oocyst counting approach after the manual dissection and mounting of mosquito midguts [7, 10, 11].

Here, using transgenic *P. falciparum* expressing the firefly luciferase protein [12], we present a luminescence based SMFA that eliminates the need for mosquito dissections. Our results from 68 separate experimental mosquito feeds (comprising data from 6860 mosquitoes), indicate that the luminescence assay performed on whole homogenised mosquitoes is equally or more sensitive than microscopy for infection detection, and that the mean luminescence intensity of groups of individual or pooled mosquitoes correlates closely with mean oocyst intensity in separate groups of mosquitoes from the same experimental feeds.. Although the luciferase assay may have limitations in accurately quantifying oocyst densities in individual mosquitoes, we propose that the luciferase-based SMFA can replace microscopy as a more efficient and objective screening technique for *Plasmodium* TRA.

Methods

P. falciparum (NF54HT-GFP-luc) culture

Transgenic *P. falciparum* gametocytes (NF54HT-GFP-luc) [12] expressing a fusion of GFP and firefly luciferase protein (14 day culture; 0.3-0.5% gametocytes and 2% haematocrit) were obtained from an automated tipper system and prepared with packed red blood cells as previously described [12-14]. The NF54HT-GFP-luc parasite line is available upon request and has been deposited at the Malaria Research and Reference Reagent Resource Center (MR4).

Mosquito breeding and feeding

Anopheles stephensi mosquitoes (Sind-Kasur Nijmegen strain) [15] were reared at 30°C and 70-80% humidity, while exposed to a 12/12-hour day/night cycle. Mosquitoes aged 3-5 days were fed on a glass membrane midi-feeder system containing 1.2 mL of the *P. falciparum* culture/reagent mix [13, 14]. Unfed and partially fed mosquitoes were removed after feeding and blood fed females were maintained at 26°C and 70-80% humidity.

Sixty-eight separate experimental mosquito feeds with *A. stephensi* (hereafter, 'feeds') were performed in 10 sets of experiments. Of these 68 feeds, 63 were performed as assessments of TRA using test or control agents; the remaining 5 were performed for assay optimisation of the luminescence assay (full details of all experiments and sample sizes in **Supplementary Table 1**). Two transmission reducing agents were used for assessments of TRA: (1) rat monoclonal antibody (mAb) 85RF45.1, specific to the *Plasmodium* protein Pfs48/45, a protein present on the surface of gametocytes that is necessary for zygote formation in the mosquito [16, 17]**;** and (2) human serum immunoglobulin G (IgG) purified from the blood of a European expatriate with well-documented TRA [6, 18]. 85RF45.1 mAb was diluted into human serum, added to the gametocyte/red blood cell culture mix (1.2 mL total), and provided to mosquitoes at 2.5 μg/mL (nine feeds), 1.25 μg/mL (3 feeds), 0.63 μg/mL (9 feeds), 0.31 μg/mL (2 feeds) and 0.16 μg/mL (2 feeds). Human serum was used as a control for TRA assessments in all 85RF45.1 mAb experiments (12 feeds). Subsidiary isotypic controls (non-transmission effective mAb specific to the *Plasmodium* Glutamate rich protein, GLURP) were also tested (3 feeds). Serum IgG was diluted into human serum and added to the gametocyte/red blood cell culture mix (1.2mL total) at 9 fold (3 feeds), 27 fold (3 feeds), 81 fold (3 feeds), 243 fold (3 feeds) and 749 fold (3 feeds) dilutions from a stock concentration of 9.8 mg/mL. Human serum was used as a control in all serum IgG experiments (5 feeds). Subsidiary isotypic controls (a 9-fold dilution of non-transmission effective IgG from native Dutch donors) were also tested (3 feeds). In all serum IgG feeds 125 μL of active complement from human sera (Sanquin Blood Supply, Nijmegen, Netherlands) was included in the culture mix. No significant differences were observed between isotypic and human serum controls, and TRA calculations based on either control

were highly correlated for all methods (**Supplementary Figure 1**); human serum control was used as the primary control as it was available for all experiments. All SMFAs were blinded for evaluation and analysis.

In a direct comparison of infection rates in *A. stephensi* and *Anopheles gambiae*, we observed no oocysts and no mosquitoes with luminescence above the positivity threshold in *A. gambiae*, confirming the role of Pf47 (disrupted during insertion of the GFP-Luciferase construct in NF54HT-GFP-Luc) in *P. falciparum* immune evasion [19] (**Supplementary Table 1**).

Microscopy and luminescence based SMFA evaluation

Individual mosquitoes were analysed separately for microscopy-based (n=30-58) and luminescence-based assessments (n=30-50) from every feed. Alongside individual mosquitoes, 3 separate groups of 5 and 10 mosquitoes were taken for processing as pools in the luminescence assay. If fewer than 3 pools of 5 or 10 mosquitoes were available because of mosquito mortality, pool-based analysis was not conducted. In total, 3 pools of 5 and 10 mosquitoes were taken from 62 and 57 feeds respectively.

For standard microscopy, live mosquitoes were collected on day 7 after infection, and midgut dissections were carried out in 1% mercurochrome solution by expert microscopists.

All luminescence assays used to assess the association of luminescence- and microscopy-based measures of infection and TRA were conducted on day 8 after infection (**Figure 1A**). Day 9 after infection was equally or more suitable for luminescence assays but was not chosen in the current study for logistical reasons. Full protocols for the luminescence assay are in the **Supplementary Materials**. Briefly, for the individual mosquito assay, live immobilised mosquitoes were homogenised for 5 seconds using re-usable plastic pestles and a hand held pestle rotator (Argos Technologies, Elgin, IL) in micro-centrifuge tubes containing 48 µL of a phosphate buffered saline (PBS, 1%, pH 7.2), 1% EDTA, 1% protease inhibitor cocktail solution (Protease Inhibitor Cocktail Kit [78410], Thermo Scientific, Waltham, MA). Individual mosquito homogenates (48 µL) were pulsed in a micro centrifuge and stored at 4°C before being mixed, pipetted into 96 well plates (uClear black plates [655090], Greiner BioOne, Frickenhausen, Germany) with 12 µL of lysis buffer (Luciferase assay system [E1501], Promega, Madison, WI), and left for 30 minutes at room temperature. After 30 minutes 60 µL of luciferase substrate was added to all wells, plates were shaken for five seconds, and bioluminescence (measured as relative light units [RLU; i.e, counts per second]) was measured using a multipurpose plate reader (Synergy 2, Biotek UK) for each well (1 second, top-down read).

For pooled mosquito luminescence assays, groups of 5 or 10 mosquitoes were homogenised as described but for 10 or 15 seconds, respectively, in 100 µL of the same PBS/Protease inhibitor mix. The volume of the homogenate was then increased to a level equal to 48 µL per mosquito (i.e., pools of 5 mosquitoes had a final volume of 240 µL,

and pools of 10 mosquitoes had a final volume of 480 μL). All pooled homogenates were mixed, and three 48 μL samples were tested by the luminescence assay as described above. The luminescence intensity of a pool of mosquitoes was determined by taking the mean of 3 separate assays sampled from the same mixed homogenate. The mean pool-associated luminescence intensity for experimental feeds was calculated as the mean value for 3 separately processed pools (yielding nine total assays per feed for a specific pool size).

Uninfected negative control mosquitoes were processed in all experimental runs (n = 23-53) for the calculation of luminescence based infection prevalence. Cut-offs were determined arbitrarily as the mean luminescence intensity of uninfected controls plus 5 SDs (**Supplementary materials**). The mean luminescence intensity of uninfected control mosquitoes was 2.5 RLU (range, 0-7 RLU), which gave thresholds for NF54HT-GFP-luc infection for the 10 different experiments of 5.7-10.1 RLU. Grouping all the uninfected controls from the ten experiments together gave a cut-off of 9 RLU. As infection prevalence was identical when using either the experiment specific or overall cut-off, the single cut off of 9 RLU was used for ease of analysis.

Data analysis

Statistical analyses were conducted by comparing separate groups of mosquitoes sampled from the same feeds. Comparisons between oocyst- and luminescence-based measures of mosquito infection prevalence were made with Chi-squared tests with Bonferonni correction. Associations between oocyst and luminescence intensity were quantified with linear regression, and differences between different luminescence-based assay outcomes were determined by Wilcoxon sign rank tests. 95% confidence intervals (CIs) around mean luminescence and oocyst intensity data were calculated by bootstrapping (1000 repetitions).

Luminescence- and microscopy-based TRA estimates from individual mosquitoes were made using generalised linear mixed models (GLMMs) with zero-inflated negative binomial error structure [5, 20]. The association of prevalence and TRA estimates made by separate methods was assessed with linear regression, with R^2 calculated against a perfect linear trend (x=y). As only 3 groups of mosquitoes were taken from each feed for pool based assessments, TRA estimates from pools of mosquitoes were calculated as the percentage reduction in mean luminescence intensity between test and control pools (**Supplementary Materials**), with 95% CIs around these estimates calculated by bootstrapping (1000 repetitions). TRA estimates less than 0% where converted to 0% for all figures.

For the analysis of TRA according to antibody dilution, a 4-parameter non-linear regression analysis was performed (**Supplementary Materials**). Statistical analysis was conducted using R (Foundation for Statistical Computing, Vienna, Austria), STATA 12 (StataCorp., TX, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA).

Results

Luminescence assays were conducted on day 8 after infection when the luciferase signal allowed better differentiation of positive and negative mosquitoes than on day 7 after infection (**Figure 1A**), plausibly as a consequence of sporozoite/luciferase proliferation in infected mosquitoes [8]. **Figure 1B** shows that the overall distribution of oocyst and luminescence intensities from individual mosquitoes are indistinguishable [12]. Overall, averaged measures of infection intensity were taken from a total of 2079 mosquitoes dissected for oocyst detection, 2141 mosquitoes processed individually in the luminescence assay, and 2640 mosquitoes assayed in pools of 5 (n = 930) or 10 (n = 1710). Mean oocyst intensity in control infections ranged from 1.7 to 47.8, and mean luminescence intensity in control infections ranged from 40.7 to 3263.9 RLU. Direct comparisons between oocyst counts and luminescence intensity from the same mosquito midguts were reported in Vaughan et al. [12]. In a small experiment, we determined oocyst counts by microscopy before removing and homogenising the examined guts with the carcass of an uninfected mosquito. Five mosquitoes with oocysts counts of 1, 8, 13 15 and 33 gave RLUs of 38, 522, 579, 737 and 1696, respectively.

Microscopy and luminescence based estimates of infection prevalence

Microscopy- and luminescence-based mosquito infection prevalence estimates were 64.9% (1350/2079) and 67.7% (1449/2141), respectively. The median luminescence intensity of negative test mosquitoes (692/2141) was 2 RLU (interquartile range [IQR], 2-4 RLU; range 0-8 RLU). As 14.1% of microscopically examined mosquitoes (190/1350) had single oocyst infections, and 43.2% of infections (583/1350) had 1-5 oocysts, the close association between oocyst- and luminescence-based prevalence estimates for all separate feeds (**Figure 1C**) strongly indicates that the luminescence assay consistently discriminated between infected and uninfected mosquitoes at all levels of infection intensity. Changing the luminescence based cut-off from the negative control mean plus 5 SDs to 3 or 10 SDs had limited impact and altered prevalence estimates to 68.8% (1474/2141) and 66% (1414/2141), respectively. Differences between luminescence- and oocyst-based prevalence estimates were non-significant for all feeds (**Figure 1C**).

Microscopy and luminescence based estimates of infection intensity

The luminescence assay accurately predicts mean oocyst intensity, despite the inherent variability in oocyst counts between separate mosquito samples from the same feeds [5, 21] (**Figure 1D**). The mean luminescence intensity of pools of 5 and 10 mosquitoes from the same feeds were also strongly associated with mean oocyst intensity (**Figure 1E**). Although the mean luminescence intensity of pooled mosquitoes was generally somewhat lower than that for individual mosquitoes (**Figure 1F**), paired luminescence

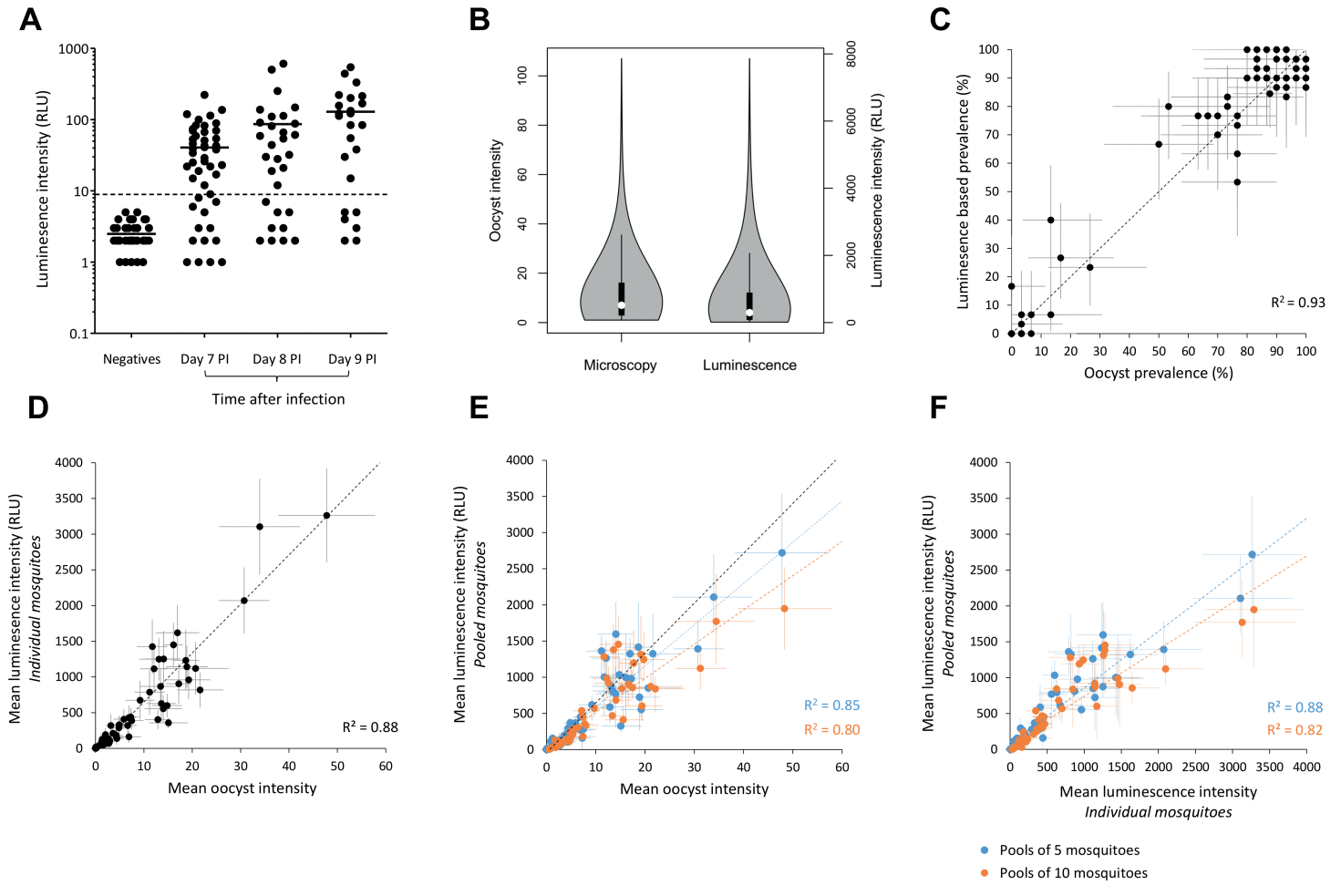
A
Luminesence intensity (RLU)
1000
100
10
1
0.1
Negatives
Day 7 PI
Day 8 PI
Day 9 PI
Time after infection
B
Oocyst intensity
Luminescence intensity (RLU)
Microscopy
Luminescence
C
Luminesence based prevalence (%)
Oocyst prevalence (%)
R² = 0.93
D
Mean luminesence intensity (RLU)
Individual mosquitoes
Mean oocyst intensity
R² = 0.88
E
Mean luminescence intensity (RLU)
Pooled mosquitoes
Mean oocyst intensity
R² = 0.85
R² = 0.80
F
Mean luminescence intensity (RLU)
Pooled mosquitoes
Mean luminescence intensity
Individual mosquitoes
R² = 0.88
R² = 0.82
Pools of 5 mosquitoes
Pools of 10 mosquitoes

Figure 1. Microscopy- and luminescence-based assessments of NF54HT-GFP-luc infection prevalence and intensity. A. Luminescence intensity values for individual mosquitoes from a single experimental feed sampled on days 7, 8 and 9 after infection. Oocyst prevalence determined by microscopy at day 7 after infection was 70% (95% confidence interval [CI], 55.4-82.1%; 35/50) with mean oocyst intensity among infected mosquitoes of 2.4 (range, 1-5). Oocyst prevalence determined by luminescence intensity was 71.4% (95% CI 56.7-83.4%; 35/49) on day 7 after infection, 70% (95% CI 50.6-85.3%; 1/30) on day 8 after infection, and 75% (95% CI 53.3-90.2%; 18/24) on day 9 after infection. The dotted line represents the positivity threshold of 9 relative light units (RLU). **B.** Violin plots showing the relative distribution of oocyst counts (n = 2079) and luminescence measurements (n = 2141) from individual mosquitoes across all experimental feeds. The central box plots show the median and interquartile range of oocyst and luminescence intensity data, and the distribution and range of data points is displayed in the surrounding rotated kernel density plots. **C.** Infection prevalence in all 68 mosquito feeds determined by microscopy and luminescence assay. Error bars represent 95% CIs. **D**. Mean oocyst intensity and mean luminescence intensity for 68 feeds (30-58 mosquitoes examined per feed; y= 68.71x - 37.68). In all figures of infection intensity, error bars represent bootstrapped 95% CI. **E.** Mean oocyst intensity from individual microscopy assessments and mean luminescence intensity of pools of 5 (print: light grey; online: blue; n=62) and 10 (print: dark grey; online: red; n=57) mosquitoes. Trend lines were calculated using linear regression on mean estimates (pools of 5 [print: light grey, single dash and dot; online: blue dash]: y = 57.0x + 17.1; pools of 10 [print: dark grey, single dash and two dots; online: red dash]: y= 47.7x + 55.3). The trend line from panel D for individual mosquitoes is also included for comparison. For data presentation, 0.5 oocysts were added on the x-axis to all data points for pools of 10 mosquitoes to distinguish between CIs from pools of 5 mosquitoes. **F.** Mean luminescence intensity from individual mosquitoes and mean luminescence intensity of pools of 5 and 10 mosquitoes. Pool data and linear regression are as in panel E**.** For data presentation, 25 RLU were added on the x-axis to all data points for pools of 10 mosquitoes to distinguish between CIs from pools of 5 mosquitoes.

values for separate feeds were not significantly different between pooled and individual mosquitoes. The mean luminescence intensity of all individually assayed mosquitoes was 470.4 RLU, while those of all pools of 5 and 10 mosquitoes were 472.5 RLU and 462.8 RLU, respectively.

Variation in luminescence intensity between repeat measures from the same pools (i.e., the intrapool variation) was low, and although sampling error might be expected to have had a major impact on interpool luminescence variation (each of the 3 pools taken from each feed represented the average luminescence intensity of five or ten mosquitoes, rather than the standard 30 mosquitoes) interpool variation was also modest, with no clear association with mean oocyst intensity (**Supplementary Figure 2**).

Assessments of TRA

TRA estimates based on oocyst counts and luminescence measures were highly related (**Figure 2A**). Deviations from this linear trend were more apparent when both methods estimated TRA at <60%, as reflected by the size of the CIs.

A stochastic element in measuring TRA is well appreciated [5, 6, 22] and a cut-off for reproducible TRA results of >80% is commonly used [6, 23]. Where TRA ≥80% was measured by either method the mean difference between luminescence and microscopy based TRA estimates was 4.0%, and CIs were equally precise for both methods. Across the range of TRAs, CIs were generally wider for luminescence (mean interval, 20.4 percentage points) than for microscopy (mean interval, 15.0 percentage points). TRA estimates based on the luminescence assay for pooled mosquitoes were similarly representative of microscopy-based estimates (**Figure 2B**). When test agents caused a TRA ≥80%, the mean difference between microscopy- and pool-based TRA estimates was 3.6% and 3.0% for pools of 5 and 10, respectively. **Supplementary figure 2A** shows the results of all pool-based TRA estimates relative to microscopy values if only single pools are used in their calculation (instead of all 3).

In our large set of blinded experiments, we determined the TRA of different blocking and non-blocking concentrations of 85RF45.1 mAb and serum IgG. TRA was calculated using microscopy and luminescence of individual mosquitoes and pools of mosquitoes. We observed excellent agreement between TRA estimates (**figures 2C and 2D)**, with only one of the weakest dilutions of serum IgG luminescence giving non-significantly higher TRA estimates derived from pools of 5 mosquitoes.

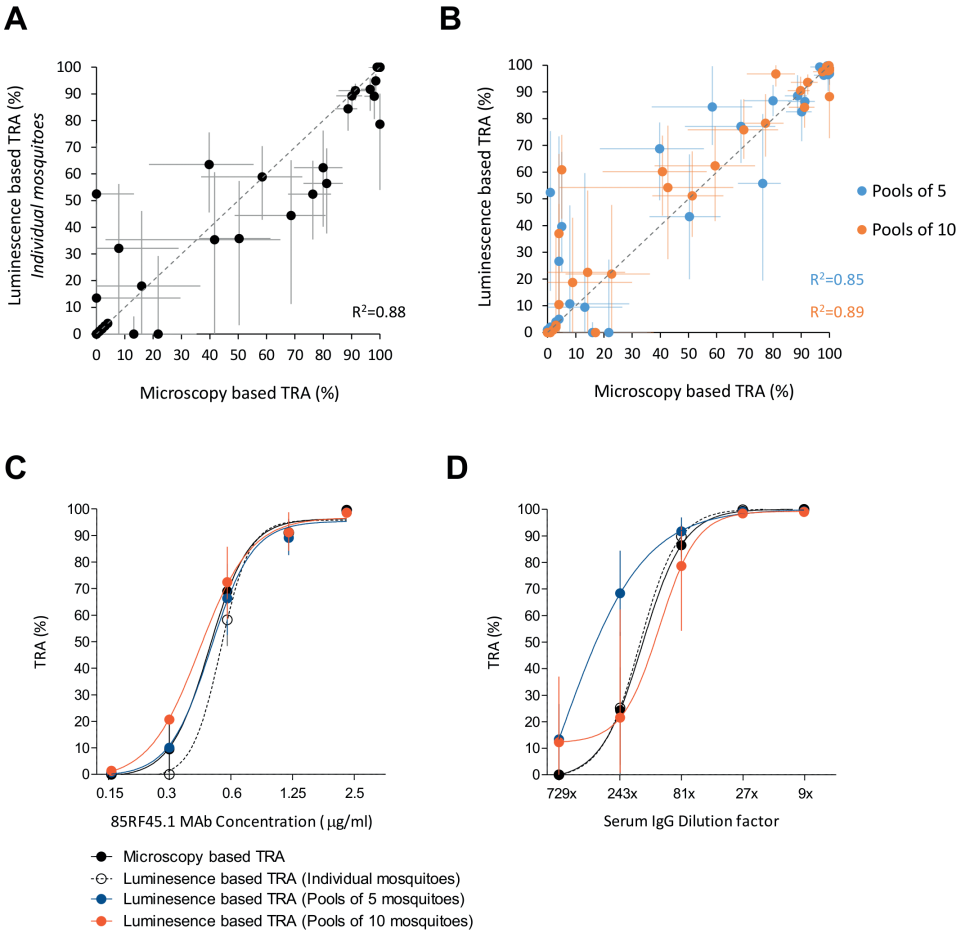

Figure 2. Microscopy- and luminescence-based assessments of the transmission-reducing activity (TRA) of transmission effective mAb (Pfs45.1), and serum immunoglobulin G (IgG). All TRA calculations were made using human serum controls (**Supplementary Figure 1**) **A.** TRA calculated separately, using mean oocyst intensity and mean luminescence intensity from individual mosquitoes from all feeds in which a transmission-reducing agent was used (n=46). R^2 was calculated based on deviation from a perfect linear association (i.e. x=y). The mean widths of the 95% CIs in which either estimate was ≥80% was 3.86 percentage points and 3.61 percentage points for luminescence and microscopy, respectively. **B.** TRA calculated separately using mean oocyst intensity and mean luminescence intensity from pools of 5 (print = light grey, online = blue) and 10 (print = dark grey, online = red) mosquitoes from all feeds for which a transmission-reducing agent was used and from which the minimum number of pools were analysed (pools of 5, n=33; pools of 10, n=38). R^2 was calculated based on deviation from a perfect linear association (i.e. x=y). For data presentation, 1% TRA was added on the x-axis to all points of pools of 10 mosquitoes to distinguish between CIs from pools of 5 mosquitoes. **C.** Non-linear regression of TRA assessments from all luminescence and oocyst intensity data from feeds with Pfs45.1 mAb. (R^2: microscopy, 0.99; luminescence for individual mosquitoes, 0.99; luminescence for pools of 5, 0.94; luminescence for pools of 10, 0.93). **D.** Nonlinear regression of TRA assessments from all luminescence and oocyst intensity data from feeds with transmission-effective serum IgG from a Dutch ex-patriate donor (R^2: microscopy, 1.0; luminescence for individual mosquitoes, 1.0; luminescence pools of 5, 0.92; luminescence pools of 10, 0.83).

Discussion

Our primary finding is that luciferase-expressing *P. falciparum* strains can be used effectively in the SMFA to provide an objective, scalable, and accurate measure of infection intensity in groups of individual and pooled mosquitoes. The luminescence assay discriminated accurately between infected and uninfected mosquitoes. The mean luminescence intensity of individual and pooled undissected mosquitoes correlated strongly with mean oocyst intensity in the same feeds, and the strength of this relationship was reflected in the close association of TRA estimates made by both methods.

Precise estimates of TRA depend on experiment replication and an adequate number of mosquito observations [5, 6]. These requirements are often not met in the SMFA as it is routinely conducted; improvements in throughput and readout are needed to allow more-robust and more-scalable assessments of TRA. We present the first approach that eliminates the requirement for mosquito dissections and allows groups of individual or pooled mosquitoes to be examined for the average intensity of infection. Previous attempts to increase SMFA efficiency continued to rely on manual mosquito dissection and semiautomated oocyst visualisation, with modest impact on SMFA throughput [7, 8]. Accepting the loss of information at the individual mosquito level, we show that the averaged luminescence of groups or pools of mosquitoes provides an accurate proxy for mean infection intensity. The most scalable approach presented in the current study was the screening of pools of mosquitoes. Although luminescence intensity was generally slightly lower for pools than for individual mosquitoes, possibly because of the slightly longer homogenisation required during pool processing, luminescence intensity was similarly predictive of oocyst intensity for all methods. When examining 3 pools from each feed and assaying each pool in triplicate, 10 feeds can be evaluated simultaneously on a single assay plate. We observed concordant results if single pools of mosquitoes were examined**,** indicating that mosquito homogenisation can be made more scalable if fewer pools are examined per feed. Further improvements in throughput and in the standardisation of mosquito processing might be achieved if pools are homogenised using plate-based bead beating (**Supplementary Materials**); this technique might also be used to scale-up individual mosquito processing if details of infection prevalence are desirable. Other clear benefits of luminescence-based outcomes are that they are recorded objectively and stored automatically. With the combined advantages of the luminescence assay we estimate a five to ten fold increase in our SMFA throughput capacity (**Supplementary Materials**).

The luciferase assay can detect increases in oocyst size and maturation, as indicated by the higher luminescence intensity on days 8 and 9 after infection, compared with that on day 7. Inevitable variations in the productivity of concomitant oocysts limit our ability to accurately interpret oocyst number from an indirect measure of luminescence intensity for individual mosquitoes, which is likely only possible using visualisation techniques [7].

However, we find no evidence that these variations affect luciferase-based TRA estimates, which consistently mirror those based on microscopical read-outs. Because size differences are more likely in high-intensity infections, where nutrient and space competition may be more pronounced, very high control oocyst intensities may need to be avoided to ensure an accurate correlation between mean luminescence and oocyst intensity. It could be argued, though, that because luciferase production reflects sporozoite proliferation more directly, luminescence intensity may provide a less abstract measure of TRA than oocyst intensity. The disadvantages of the traditional SMFA in this respect have been discussed previously [5, 8]. Luminescence-based outcomes are particularly suitable for studies where changes in oocyst size or maturation and not solely their presence are of interest [8] and for assessments of other aspects of sporogonic development, such as haemolymph or salivary gland infection, where parasite loss during standard investigations is a concern.

In the conventional SMFA with microscopy-based readouts the threshold for transmission blockage is commonly set at 80% transmission reduction [23], not because vaccines or drugs with lower levels of TRA may not be of value [24] but because there is considerable variation in estimates of low level TRA, and only high level transmission reduction can be reproducibly measured [6, 22]. As anticipated, in our experiments, CIs were narrowest and agreement between luminescence- and microscopy-based estimates were greatest in the transmission blocking range. Estimating lower levels of the TRA requires a larger number of mosquito observations, ideally collected through a study in which the activity of compounds or test sera is assessed against a range of control oocyst densities[5]. Because of its scalability and strong association with oocyst prevalence and intensity, the proposed luminescence assay can facilitate these repeated assessments of large mosquito numbers that may be operationally unattractive when relying on microscopy. Despite the value of our approach, there are experiments where microscopy remain of indisputable value. These include experiments with *A. gambiae* mosquitoes, where the use of the NF54HT GFP-Luc parasite line is precluded, and investigations where oocyst enumeration is of specific interest.

A high-throughput, semiautomated SMFA could significantly increase the efficiency of screening drugs and vaccines with potential effects on *Plasmodium* transmission, which have taken a central role in plans to achieve malaria eradication. The results of the current study represent a scalable alternative to traditional SMFA techniques that may also increase the operational feasibility of population-level trials with transmission-blocking vaccines, by complementing direct-feeding assessments with scalable assessments of transmission blocking immunity [25].

Acknowledgments

We would like to thank Jacqueline Kuhnen, Laura Pelser-Posthumus, Astrid Pouwelsen, and Jolanda Klaassen for mosquito breeding, and for their assistance during SMFA evaluation. The work of WS, KL, and TB is supported by grants from the Bill & Melinda Gates

Foundation (AFIRM, grant number OPP1034789) and PATH Malaria Vaccine Initiative (MVI). TB is further supported by European FP7 project REDMAL (#242079) and a Marie Curie Career Integration Grant from the European Community's Seventh Framework Programme (SIGNAL, PCIG12-GA-2012-333936). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Selected supplementary material

Supplementary Tables 1 A. Experiments performed for the primary analyses of luciferase and oocyst based measures of NF54HT-GFP-Luc infection and TRA. **B.** Experiments to determine the permissiveness of *A. stephensi* and *A. gambiae* to NF54HT-GFP-Luc. Three small experiments were conducted to confirm the role of *Pf47* in *Plasmodium* immune evasion during *A. gambiae* infection. *A. stephensi* and *A. gambiae* mosquitoes were infected with three gametocyte cultures, and 30 mosquitoes from each of these feeds were assayed individually for oocyst and luminescence intensity. Whilst notable, there are at present no indications of vector specific transmission reducing effects of malaria drugs or vaccines, suggesting that the proposed SMFA, though restricted to *A. stephensi,* would have broad utility. **C.** Preliminary experiments to determine the effect of bead beating for mosquito homogenization on luminescence intensity. Four pools of ten mosquitoes from separate experiments processed using standard methods and plate-based bead beating to determine the effect on luminescence intensity of high-throughput mosquito pool grinding. Standard homogenisation was for 15 seconds with a hand-held pestle rotator. The impact of bead beating was determined by subjecting the same standard homogenate to an additional 10 second high-intensity homogenisation with zirconia beads in deep-well plates, as described in the full protocol.

A.

SMFA Experiment ID	Test feeds	Control feeds	Feeds total	Test antibody type	Day of luminescence assay (PI)	Microscopy		Luminescence assay (number of mosquitoes/pools per feed)					
						Dissected	*n*	Individuals	*n*	Pools of 5	*n*	Pools of 10	*n*
1	-	2	1	-	7, 8 & 9/8	50/49	99	103/58	161	-	-	-	-
2	4	2	6	45.1 Mab	8	30	180	30	180	3	90	0/3	90
3	4	2	6	45.1 Mab	8	30	180	30	180	3	90	3	180
4	4	2	6	45.1 Mab	8	30	180	30	180	0/3	60	-	-
5	4	2	6	45.1 Mab	8	30	180	30	180	0/3	75	3	180
6	6	2	8	45.1 Mab	8	30	240	30	240	3	120	3	240
7	6	2	8	45.1 Mab	8	30	240	30	240	3	120	3	240
8	6	2	8	TB serum IgG	8	30	240	30	240	3	120	3	240
9	6	1	7	TB serum IgG	8	30	210	30	210	0/3*	90	3	210
10	6	5	11	TB serum IgG	8	30	330	30	330	3	165	3	330
Total feeds/cages	46	22	68										
Total mosquitoes							**2079**		**2141**		**930**		**1710**

SMFA Standard membrane feeding assay
TB Transmission blocking
PI Post-infection
N Total mosquito sample size per feed (individually assayed or assayed as pools)
0/3 Zero pools assayed for some feeds in experiment (due to insufficient pool number)
* No control pools of five in experiment 9, therefore no TRA based analysis was performed.

B.

Measure of infection	Experiment	Mosquito species	Mean	25th percentile	75th percentile
Oocysts	1	***St.***	47.8	22.5	69.0
		Ga.	0.0	0.0	0.0
	2	***St.***	33.9	16.0	53.8
		Ga.	0.0	0.0	0.0
	3	***St.***	20.7	4.3	35.5
		Ga.	0.0	0.0	0.0
RLU	1	***St.***	3263.9	1726.3	4640.3
		Ga.	1.9	1.0	3.0
	2	***St.***	3105.4	1511.5	4152.3
		Ga.	2.2	0.8	3.0
	3	***St.***	1120.1	256.8	1746.5
		Ga.	3.1	2.0	4.0

RLU Luminescence intensity (relative light units)
St. *A. stephensi*
Ga. *A. gambiae*

C.

Pool/Feed	Mean luminescence intensity (RLU)	
	Hand grinding	***Bead beating***
1	152.5	148
2	18.5	13.5
3	240	210
4	107	112.5

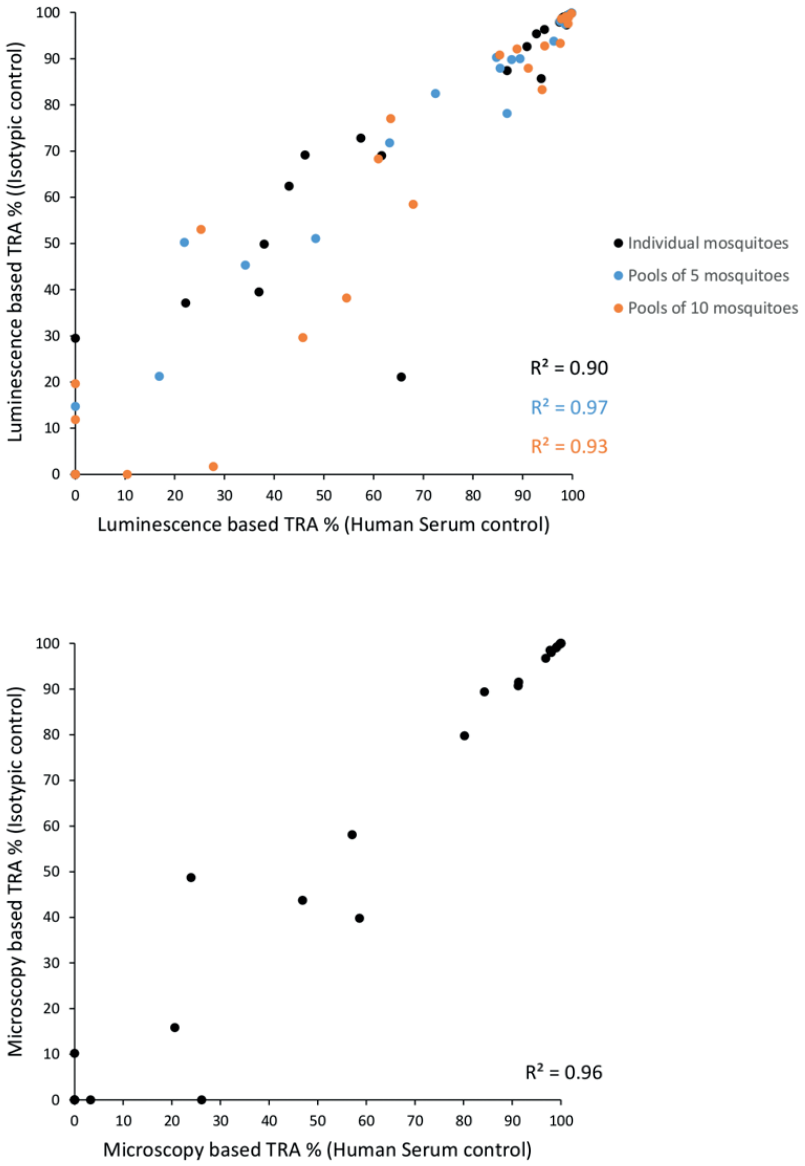

Figure S1 TRA estimates made using human serum and isotypic controls. X-axis = TRA calculated using human serum controls. Y-axis = TRA calculated using IgG or MAb isotypic controls. TRA was calculable using isotypic controls for 28 of the 46 experiments in which any transmission reducing agent was applied.

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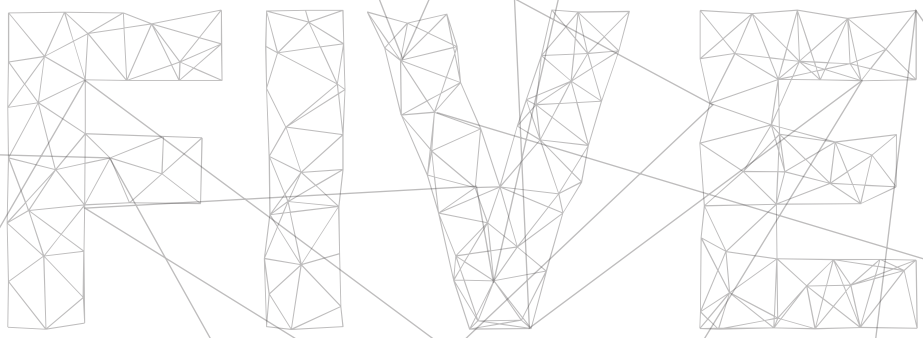
FIVE

Chapter 5b

A scalable assessment of *P. falciparum* transmission in the standard membrane-feeding assay, using transgenic parasites expressing Green fluorescent protein–Luciferase

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Methods in Molecular Biology. 1325: Springer New York; 2015. p. 101-12

Abstract

In pre-clinical development, the efficacy of agents with putative effects on *Plasmodium* transmission is determined using the standard membrane feeding assay (SMFA). Because the end-point of the SMFA is normally the enumeration of oocysts on the mosquito midgut, the assays reliance on mosquito dissections and microscopy makes it slow, labour intensive, and subjective. Below, we describe a novel method of assessing the transmission of a *Plasmodium falciparum* strain expressing the firefly luciferase protein in the SMFA. The use of a transgenic parasite strain allows for the elimination of mosquito dissections in favour of a simple approach where whole mosquitoes are homogenised and examined directly for luciferase activity. Measuring the mean luminescence intensity of groups of individual or pooled mosquitoes provides comparable estimates of transmission reducing activity at 5-10 fold the throughput capacity of the standard microscopy based SMFA. This high efficiency protocol may be of interest to groups screening novel drug compounds, vaccine candidates, or sera from malaria exposed individuals for transmission reducing activity (TRA).

Introduction

Interventions that reduce the likelihood of humans with infectious malaria parasites seeding secondary mosquito infections are valuable components of malaria elimination campaigns [1, 2]. All drugs or vaccines that affect the asexual reproduction of *Plasmodium* life stages supress transmission by limiting the number of parasites that develop into gametocytes; the life stages responsible for transmission from humans. In addition, drugs and vaccines can also specifically target mature transmission stages, gametocytes, or their transmissibility to mosquitoes. Only a limited number of antimalarial drugs have activity against gametocytes; these drugs include artemisinin derivatives [3], 8-aminoquinolines [4], and methylene blue [5]. With the renewed interest in malaria elimination, testing the activity of novel candidate drugs on gametocytes and their transmissibility is now widely advocated [6-9].

The development of transmission blocking vaccines (TBVs) is supported by the identification of immune markers of transmission reducing activity (TRA) among naturally exposed populations [10-15]. Antibodies to a handful of antigens expressed during the parasites sporogonic cycle in the mosquito have been shown to be able to interrupt this development to prevent mosquito infectivity [16], although it is evident that the full immune profile of naturally acquired transmission-reducing immunity remains to be explored.

The effect of any agent on *Plasmodium* transmission is determined by performing the standard membrane feeding assay (SMFA), in which mosquitoes are fed the putative transmission blocking agent along with a mixture of cultured *Plasmodium* gametocytes and blood. The outcome of the assay is the prevalence and intensity of parasites achieving successful sporogony in a sample of mosquitoes, which is determined by the quantification of *Plasmodium* oocysts on the dissected mosquito midgut. TRA is generally defined as the percentage reduction in mean oocyst intensity between test and control mosquitoes [17, 18]. Because the assay involves manually dissecting individual mosquitoes, and because of the requirement for large sample sizes to ensure precision in TRA estimates [17, 19], the SMFA in its current form is limited by labour intensiveness and subjectivity [20, 21].

Transgenic *P. berghei* variants (PbGFPCON) have been used previously to increase the scalability of the SMFA. Though excellent correlations are described between *in silico* fluorescence based and manual oocyst counts [20, 22, 23], the technique requires all mosquito mid guts to be dissected and mounted prior to counting, so remains limited in scalability. Recently, a strain of *Plasmodium falciparum* expressing a fusion of the Green Fluorescent (GFP) and firefly Luciferase proteins, NF54HT-GFP-luc, was produced by Vaughan et al. [24]. This strain can be cultured in similar conditions and shows comparable growth patterns to the standard *P. falciparum* NF54 parasite strain. Importantly, NF54HT-GFP-luc was shown to express luciferase throughout the parasites life cycle, including all stages of the parasites sexual development in the mosquito [24]. Here, using cultured NF54HT-GFP-luc gametocytes, we present a luminescence based approach to SMFA evaluation

that eliminates the need for mosquito dissections. Our previous assessments indicate that the luminescence assay performed on whole homogenised mosquitoes is equally or more sensitive than microscopy for infection detection, and that the mean luminescence intensity of groups of individual or pooled mosquitoes correlates closely with mean oocyst intensity in the same mosquito groups [25]. Variable productivity between concomitant oocysts [26, 27] limits the degree to which we can interpret actual oocyst number from luminescence intensity for individual mosquitoes. Therefore, for experiments requiring precise oocyst numbers, visualisation and counting are still essential [20]. However, we suggest that because luciferase production reflects sporozoite proliferation more directly, luminescence intensity may provide a less abstract measure of TRA than oocyst intensity. Certainly, the assay is particularly suitable for studies where changes in oocyst size or maturation and not solely their presence are of interest [21].

The procedures involved in the SMFA have been described in detail elsewhere [28, 29]. It is not our intention to describe the already standardised methods of gametocyte culture, mosquito husbandry, and mosquito infection, but to provide a scalable alternative to microscopy for the determination of infection prevalence and TRA. Below, we provide a short summary of the methods involved in each step of the SMFA, with notes on details particularly relevant to our evaluation methodology.

Experimental design

Our method involves assaying the luminescence intensity of homogenised individual or pooled mosquitoes, and utilising this measure of luciferase activity as a proxy for mosquito infection intensity. Section *3.3* of the methods describes how infection prevalence in groups of individual mosquitoes is determined using a cut-off based on the luminescence intensity of uninfected control mosquitoes. For the calculation of TRA, it is necessary to conduct separate mosquito feeds using the same gametocyte culture but with the addition of a neutral control (non-transmission blocking, and preferably isotypic). TRA may be calculated as the percentage reduction in mean luminescence intensity between each group of test mosquitoes and the control (Equation 1). This method, transplanting mean oocyst intensity with luminescence intensity, is the most commonly used method of assessing the SMFA. Alternatively, when using individual mosquitoes, TRA may also be calculated from luminescence or oocyst outcomes using generalised linear mixed models (GLMMs). GLMMs incorporate the distribution of oocyst/luminescence counts from individual mosquitoes allowing for more robust analyses of TRA, and limiting the sample size necessary for achieving adequate statistical power [17]. Such analysis is not possible using the pooled mosquito assay, as only 2/3 observations will be recorded per cage.

Equation 1.

A. Oocyst intensity based TRA

$$Microscopy\ based\ TRA\ \% = \left(\frac{[Mean\ oocyst\ intensity\ of\ control\ mosquitoes\text{-}Mean\ oocyst\ intensity\ of\ test\ mosquitoes]}{Mean\ oocyst\ intensity\ of\ control\ mosquitoes}\right) *100$$

B. Luminescence intensity based TRA

$$Luminesence\ based\ TRA\ \% = \left(\frac{[Mean\ RLU\ of\ control\ mosquitoes\text{-}Mean\ RLU\ of\ test\ mosquitoes]}{Mean\ RLU\ of\ control\ mosquitoes}\right) *100$$

With both the individual and pooled assay, the same numbers of mosquitoes should be sampled per cage as would be acceptable with oocyst detection (generally 25-50), however the increased scalability of the pooled method makes the evaluation of greater numbers of mosquitoes very feasible.

Gametocyte culture

The NF54HT-GFP-luc parasite line is available upon request, and has been deposited at the Malaria Research and Reference Reagent Resource Center (MR4). Because parasite transfection was based on single strand cross-over of the pEFGFP–luc plasmid, NF54HT-GFP-luc requires exposure to the selective agent WR99210 during culture to avoid reversion to wild type. Except for the addition of this selective agent, gametocyte culture for NF54HT-GFP-luc is no different than for the standard NF54 *P. falciparum* parasite line: Transgenic *P. falciparum* gametocytes (NF54HT-GFP-luc) (14 day culture, 0.3-0.5% gametocytes, 2% haematocrit) should ideally be obtained from an automated tipper system and prepared with packed red blood cells as previously described [24, 28, 29].

Mosquito husbandry and the SMFA

Though the insertion of the GFP-Luciferase construct into the *Pf47* gene locus of NF54HT-GFP-luc was conducted under the assertion that the Pf47 protein was 'dispensable', gene knockouts have since proven that the locus is essential for evasion of the mosquito immune system in *Anopheles gambiae,* but not in *A. stephensi* which lacks functional thioester containing protein 1 (TEP1) mediated immune mechanisms [30]. The NF54HT-GFP-luc *P. falciparum* strain is therefore restricted to experiments with *A. stephensi*. Though *A. gambiae* is the dominant vector for *P. falciparum* in Africa, the SMFA for *P. falciparum* transmission is generally conducted using the *A. stephensi* vector system, which is easier to breed and more permissive to infection.

For the SMFA, *Anopheles stephensi* should be reared as standard (30°C and 70-80% humidity, exposed to a 12/12 hour day/night cycle). 3-5 day old mosquitoes should be fed on a glass membrane midi-feeder system containing 1.2 ml of the *P. falciparum* culture/reagent mix [28, 29]. Unfed and partially fed mosquitoes should be removed after feeding, and blood fed females should then be maintained at 26°C and 70-80% humidity. In each experiment, additional uninfected mosquitoes need to be assayed for the determination of infection positivity in test mosquitoes.

Infection detection and quantification using bioluminescence

Though oocyst detection by microscopy is possible from day 6 after initial infection [31] we advise waiting longer before conducting the luminescence assay to ensure optimal oocyst productivity. Luminescence intensity and the distinction between positive and negative mosquitoes increases from day 7 onwards [25]. Though oocyst productivity increases until their rupture at around day 10 post-infection (PI), thereafter becoming unpredictable due to sporozoite loss during hemolymph traversal, because of the increasing chance of mosquitoes becoming infectious to humans we suggest that day 9 PI is probably optimal for luminescence assessments. We suggest that the luminescence assay time point should generally be weighed on the aims of the study, but that day 7 PI is perhaps too early to sufficiently differentiate positive and negative mosquitoes.

Two variations on our standard method of luminescence based evaluation of mosquito infection are presented. These are as follows:

Individual mosquito assessments (subheadings 3.2 and 3.3): Individual mosquitoes are homogenised and assayed, allowing for the determination of infection prevalence. The mean luminescence intensity of all mosquitoes in a sample is used as a proxy for mean oocyst intensity in the calculation of TRA. Though more objective, the speed of such assessments is not much in excess of microscopic oocyst detection.

Pooled mosquito assessments (subheadings 3.3 and 3.4): For increased scalability, pools of mosquitoes may be homogenised and assayed together. The mean luminescence intensity of a pre-determined number of repeat pools from each experimental group of mosquitoes is used for the calculation of TRA. Estimates of infection prevalence are not possible when pooling, but the scalability of the TRA assessments is increased 5-10 fold, depending on chosen sample numbers. Details of the timing of the pooled assay, with comparison to microscopy, are provided in the supplementary information of Stone et al. 2014 [25]. For each experimental feed, we find that the mean luminescence intensity of 3 pools of 5 or 10 is strongly correlated with mean luminescence intensity determined in the individual mosquito assay (subheading 3.2) when these are carried out on separate mosquito samples from the same feeds. We also find that these measures provide TRA estimates that correlate strongly with TRA estimates made using standard oocyst counts.

Materials

Luminescence based SMFA evaluation

1. CO_2 source for mosquito sedation
2. Netted cages for mosquito collection
3. Auto-clavable pestles (Argos Technologies, Elgin, IL)
4. Handheld pestle rotator (Argos Technologies, Elgin, IL)
5. 1.5ml conical bottom microcentrifuge tubes
6. Grinding buffer: Phosphate buffered saline (PBS) pH 7.2, containing 1% EDTA, 1% Protease inhibitor cocktail solution (Protease Inhibitor Cocktail Kit [78410], Thermo Scientific, Waltham, MA)
7. 200 µl pipette tips
8. Tube racks
9. Ice trays
10. Microcentrifuge
11. 96 well clear bottomed plates (uClear black plates [655090], Greiner BioOne, Frickenhausen, Germany)
12. Plate rocker
13. Luciferase assay buffer (Luciferase assay system [E152A], Promega, Madison, WI)
14. Luciferase assay substrate (Luciferase assay system [E151A], Promega, Madison, WI)
15. Multi-purpose plate reader (Synergy 2, BioTek, Winooski, VT)

Methods

Preparation of reagents

1. Prepare fresh mosquito grinding buffer. Dispose of if unused after 24 hours.
2. Remove luciferase assay buffer from storage at -20°C, and allow to thaw to room temperature.

Determining infection prevalence and intensity in groups of individual mosquitoes

1. Prepare assay plate plans, including space for blank wells (assay substrates only) and negative controls on each plate. In each experimental run, at least the same number of uninfected negative control mosquitoes should be processed as for a single infectious feed (i.e. if 30 mosquitoes are processed from each infectious feed, at least 30 uninfected mosquitoes should be processed as negative controls, and assayed on the same plates. Because individual mosquito assays require numerous assay plates, uninfected controls should be spread out so that some are assayed on every plate).

2. Aliquot 48 µl of mosquito grinding buffer into microcentrifuge tubes (one tube per mosquito). Place tubes in racks (see Note 1), and store until ready for use at 4°C.
3. Aliquot 12 µl of luciferase assay buffer into all assay plate wells according to plate plans. Store covered at room temperature (see Note 2).
4. Remove required number of mosquitoes from primary feeding cage into a smaller netted container.
5. Remove microcentrifuge tubes from the fridge into an ice-tray, and move the tray next to the mosquito cage on the bench-top.
6. Immobilize mosquitoes with CO_2.
7. Remove mosquitoes one by one into microcentrifuge tubes. As soon as a mosquito is placed in a tube, place a pestle on top, with enough pressure to ensure that the mosquito is submerged and killed (see Note 3).
8. Attach a hand-held pestle rotator to the pestle, and homogenise the mosquito for approximately 5 seconds (see Note 4). Spin pestle at top of microcentrifuge tube to remove excess homogenate (see Note 5). After homogenisation, remove pestle to a container for washing and autoclaving to allow re-use.
9. Repeat for all mosquitoes (see note 6).
10. Pulse mosquito homogenates in a microcentrifuge for 15 seconds to remove residual homogenate from tube walls, and store in racks at 4°C.
11. Remove homogenates from 4°C, and using modified 200 µl pipette tips (see Note 7) mix and aliquot all mosquito homogenates separately from tubes into assay plate wells. 1-2 µl residual homogenate in the microcentrifuge tube is inevitable, but unavoidable (see Note 5). When entire assay plate is filled according to plate plan, cover and agitate gently on a rocker at room temperature for 30-45 minutes.
12. During the first plates incubation with luciferase assay buffer, reconstitute luciferase substrate according to the number of mosquitoes under analysis (60 µl per mosquito), and leave to equilibrate at room temperature in darkness (see Note 8). Additionally, ensure that the plate reader is on and warmed up, with settings appropriate for the luminescence assay (all wavelengths, 1 second measurement per well is sufficient).
13. With a multichannel pipette, add 60 µl luciferase substrate to every well (see Note 9). Place plate into plate reader immediately, shake for 5 seconds to mix (manually, if the machine is not capable of doing so during its program), and assay for luminescence intensity.

Data processing

1. Name and store files with reference to assay date and plate plans.
2. Transfer all raw data (from an entire experiment) into a combined spread sheet, and calculate summary statistics, including mean blank well luminescence (from every plate). From each plate, the mean blank well value should be subtracted from all

test well values (including uninfected mosquito controls) to correct for background luminescence.

3. A cut-off for infection positivity can be determined as the mean luminescence intensity of the corrected luminescence values for the uninfected controls (from every plate combined) plus five standard deviations (see Note 10).

Determining infection intensity in groups of pooled mosquitoes

1. Prepare assay plate plans, including space for blank wells (assay substrates only) and negative control pools on each plate. Pool size and number should be decided in advance of all experiments. Here, we describe the preparation of 3 pools of 10 mosquitoes. In previous publications we have shown that 3 pools of 5 or 10 mosquitoes give similar results (see Introduction section 1.4). In each experimental run, we suggest that at least the same number of uninfected negative control mosquitoes should be processed as for a single infectious feed (*i.e.* if 3 pools of 10 are processed from each feed, 3 pools of 10 uninfected mosquitoes should be processed as negative controls, and assayed on the same plates).
2. Aliquot 100 µl of mosquito grinding buffer into microcentrifuge tubes (one tube per pool). Place tubes in racks (see Note 1), and store until ready for use at 4°C.
3. Aliquot 12 µl of luciferase assay buffer into all assay plate wells according to plate plans. Store covered at room temperature (see Note 2).
4. Remove required number of mosquitoes from primary feeding cage into a smaller netted container.
5. Remove microcentrifuge tubes from the fridge into an ice-tray, and move the tray next to the mosquito cage on the bench-top.
6. Immobilise mosquitoes with CO_2.
7. Remove mosquitoes one by one into a microcentrifuge tube. As soon as 10 mosquitoes are placed in a tube, place a pestle on top, with enough pressure to ensure that all mosquitoes are killed (see Note 3).
8. Attach a hand-held pestle rotator the pestle, and homogenise the mosquitoes for approximately 10-15 seconds (see Note 4). Spin pestle at top of microcentrifuge tube to remove excess homogenate (see Note 5). After homogenisation, remove pestle to a container for washing and autoclaving to allow re-use.
9. Repeat for all mosquito pools (see note 6).
10. Total volume of mosquito grinding buffer per mosquito should be kept at 48 µl, as for the individual mosquito assay. After homogenisation, for pools of 10 mosquitoes, add additional mosquito grinding buffer to a total volume of 480 µl (380ul additional buffer). For pools of 5 mosquitoes, add additional mosquito grinding buffer to a total volume of 240 µl (140 µl additional buffer).

11. Pulse mosquito homogenates in a micro-centrifuge for 15 seconds to remove residual homogenate from tube walls, and store in racks at 4°C.
12. Remove homogenates from 4°C, and using modified 200 µl pipette tips (see Note 7) mix and aliquot at least 3 samples of 48 µl from each pooled homogenate into assay plate wells (see Note 5). When entire assay plate is filled according to plate plan, cover and agitate gently on a rocker at room temperature for 30-45 minutes.
13. During the first plates incubation with luciferase assay buffer, reconstitute luciferase substrate according to the number of mosquitoes under analysis (60 µl per mosquito), and leave to equilibrate with room temperature in darkness (see Note 8). Additionally, ensure that the plate reader is on and warmed up, with settings appropriate for the luminescence assay. Settings are as follows:
14. During the first plate's incubation with luciferase assay buffer, ensure that the plate reader is on and warmed up. Ensure that settings are appropriate for the luminescence assay (all wavelengths, 1 second measurement per well is sufficient).
15. With a multichannel pipette, add 60 µl luciferase substrate to every well (see Note 9). Place plate into plate reader immediately, shake for 5 seconds to mix (manually, the machine is not capable of doing so during its program), and assay for luminescence intensity.

Notes

1. When handling very large numbers of mosquitoes, it is easiest after addition of grinding buffer to microcentrifuge tubes to leave them open at 4°C. It is therefore important that they be undisturbed, to minimise the risk of contamination.
2. Luciferase assay buffer is stored at -20°C, and though it does not freeze at this temperature it is very viscous, remaining so until fully equilibrated at room temperature. Because of its viscosity, special care should be taken to ensure accuracy with pipetting. Multi-channel pipettes are useful here, but wastage in reservoirs is significant.
3. If homogenising all mosquitoes from a cage at once, we find it easiest to remove all mosquitoes to their tubes, placing a pestle into each tube in turn to immediately to kill the mosquito. Homogenisation can then be initiated at the convenience of the technician. This also avoids time wastage due to CO_2 immobilisation, which requires repetition every few minutes.
4. Manual homogenisation is not truly standardisable, however, it quickly becomes apparent how long it takes for a mosquito to become homogenised. We find that 5 seconds is long enough to ensure an even mixture when homogenising single mosquitoes, with no large body parts remaining intact. Mosquitoes differ in size, so this may differ by 1 or 2 seconds either way. Some flexibility should be allowed here, but it is important that mosquitoes are not homogenised excessively. We

have conducted studies using automated bead beating devices that show that high temperature/friction has a limiting effect on luciferase activity, which is why we advise caution.

5. For mosquito pools, longer homogenisation is necessary to achieve the same consistency. For pools of 5 we advise approximately 10 seconds homogenisation, while for pools of 10 mosquitoes, 15 seconds is adequate.
6. An additional consideration for the homogenisation process is loss of material on the pestle. After homogenising the mosquito, we spin the pestle in the empty upper half of the microcentrifuge tube to remove residual homogenate. We find that if any debris does remain on the pestle, this tends to be the harder and larger portions of the mosquito carcass (*i.e.* parts of leg). Though this, and the process of pipetting homogenates from tubes to assay wells, mean that a small amount of homogenate may not be assayed, our results on the sensitivity of this assay for the detection of low intensity infections (see Methods 3.3) indicate that this has no detrimental effect.
7. As only a portion (3 aliquots) of each pool is assayed, homogenate loss is not an issue for pooled mosquitoes, so no additional measures need to be taken after homogenisation. However, if the entire homogenate is required, the pestle may be rinsed into the same microcentrifuge tube while making the homogenate up to volume (48 µl grinding buffer per mosquito). For pools of 5 mosquitoes pestles can be rinsed with 140 µl of additional buffer, and for pools of 10 pestles can be rinsed with 380 µl. Our previous results indicate that there is remarkable consistency in luminescence read-outs between separate aliquots from the same mosquito pools [25].
8. As discussed in Note 1, and Introduction 1.4, the individual luminescence assay in the form described here does not represent a major increase in throughput over mosquito dissections. To increase scalability (for example if assessing enough mosquitoes to require 2 or more assay plates) it is generally useful if two technicians are available. After homogenisation of enough mosquitoes to fill one assay plate, a second technician can then immediately prepare these homogenates for lysis, whilst the remaining cages of mosquitoes are homogenised.
9. However, this is not essential, as the protease inhibitors in the grinding buffer are stable at room temperature for 24 hours. By ensuring that mosquitoes are stored at 4°C after homogenisation, a single technician could perform homogenisation and the assay sequentially, over a suitable timeframe.
10. Because homogenates contain mosquito debris, pipetting using standard tips is not efficient (blockage is common, and leads to wastage of the homogenate, and time). Before beginning the assay, whenever convenient, we find it useful to cut the first half centimetre off a group of 200 pipette tips, to widen the opening and allow unimpeded transfer of mosquito homogenates.

11. Another option would be to spin down the mosquito homogenates to form a pellet, then remove only the supernatant using standard pipette tips to the assay plate. We find this works equally well, but in the interests of time and standardisation have more often chosen to use the whole mixed homogenate.
12. Luciferase substrate should be kept in total darkness except during pipetting, and should ideally be used on the day of reconstitution (though freezing once for re-use is acceptable).
13. Because of the time sensitive reaction of the Luciferase enzyme once in contact with substrate, a multichannel pipette is essential here. Pipetting should be left until immediately prior to measurement. With a 1 second read per well, the time between substrate addition and assaying all wells in a 96 well plate should be just over 2 minutes. This is well within the stability range of the substrate we advise use of here. Other luciferase assay systems may have longer stability.
14. Previous results indicate that infection detection using individual mosquitoes is exceptionally robust at all levels of infection intensity. In experiments where almost half of all oocyst positive mosquitoes were in the 1-5 oocyst range (n=1350), prevalence estimates made by microscopy and luminescence assay from separate mosquito samples were very similar (64.9% and 67.7% respectively), and adjustment of the luminescence based positivity threshold between the mean plus 3 – 10 SD had little impact on these prevalence estimates (66-68.8%) [25].

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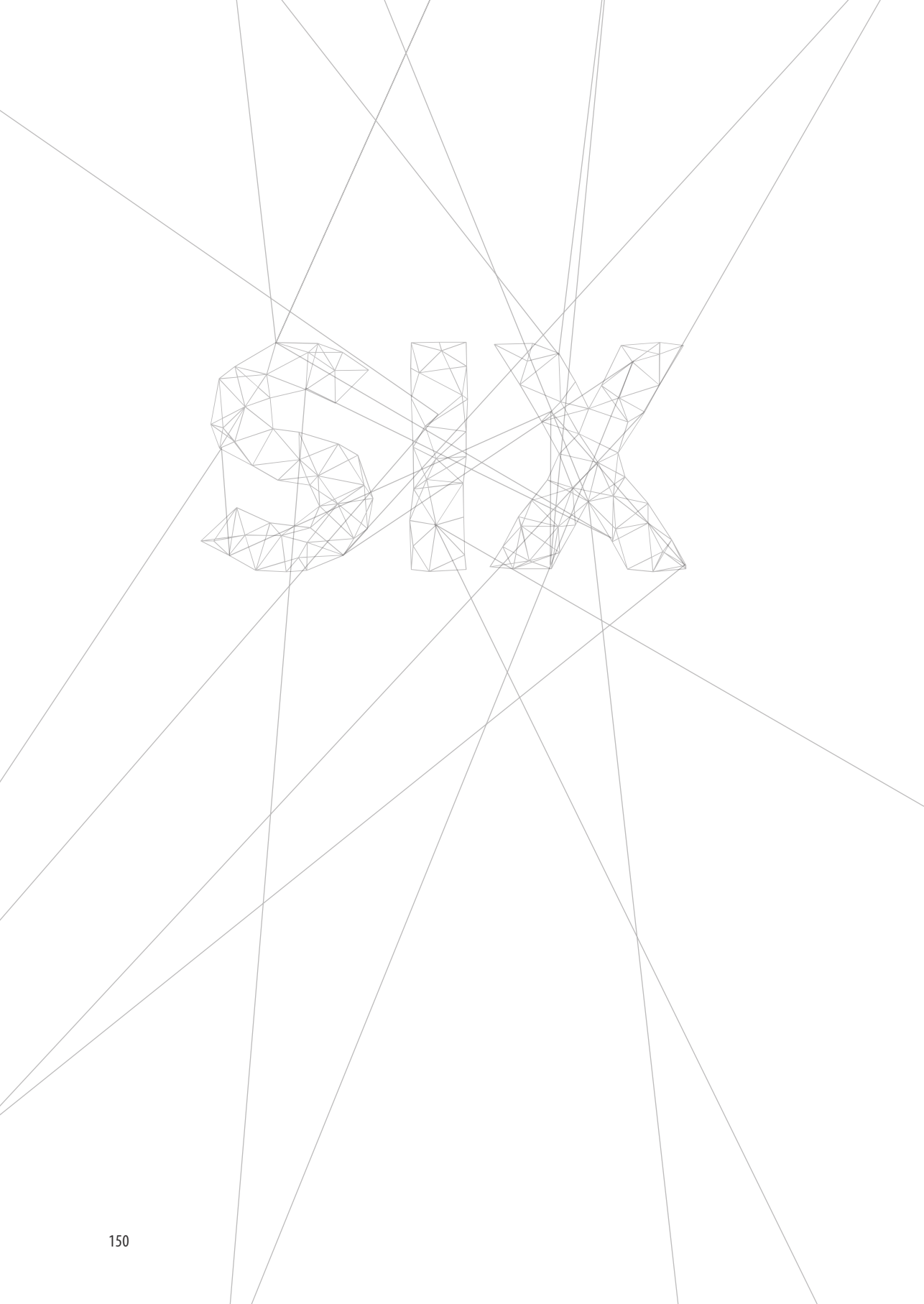
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Chapter 6

A semi-automated luminescence based standard membrane feeding assay identifies novel small molecules that inhibit transmission of malaria parasites by mosquitoes

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Scientific Reports. 2015; 5: 18704

Abstract

Current first-line treatments for uncomplicated *falciparum* malaria rapidly clear the asexual stages of the parasite, but do not fully prevent parasite transmission by mosquitoes. The standard membrane feeding assay (SMFA) is the biological gold standard assessment of transmission reducing activity (TRA), but its throughput is limited by the need to determine mosquito infection status by dissection and microscopy. Here we present a novel dissection-free luminescence based SMFA format using a transgenic *Plasmodium falciparum* reporter parasite without resistance to known antimalarials and therefore unrestricted in its utility in compound screening. Analyses of sixty-five compounds from the Medicines for Malaria Venture validation and malaria boxes identified 37 compounds with high levels of TRA (>80%); different assay modes allowed discrimination between gametocytocidal and downstream modes of action. Comparison of SMFA data to published assay formats for predicting parasite infectivity indicated that individual *in vitro* screens show substantial numbers of false negatives. These results highlight the importance of the SMFA in the screening pipeline for transmission reducing compounds and present a rapid and objective method. In addition we present sixteen diverse chemical scaffolds from the malaria box that may serve as a starting point for further discovery and development of malaria transmission blocking drugs.

Introduction

The introduction of Artemisinin combination therapy (ACT) as the first line treatment for malaria has contributed substantially to recent declines in child mortality across Africa [1, 2]. ACT rapidly clear the *Plasmodium* life stages responsible for clinical disease, but have only limited effect against mature gametocytes, the stage transmitted to mosquitoes [3]. Though immature gametocytes may be cleared shortly after their production [4] resulting in reduced gametocyte burden and transmission potential [5], current dosing regimens of ACT do not radically clear mature transmission stages and do not prevent transmission shortly after treatment [6]. Expanding efforts to achieve local malaria elimination and contain transmission in areas of emerging ACT resistance have stimulated interest in combining standard treatment with compounds active against mature gametocytes [7] and have highlighted the need to screen new antimalarial drug candidates for their transmission reducing activity (TRA).

Currently the only available drug that radically prevents malaria transmission is primaquine [8], but its wide-scale deployment is hampered by issues of safety and dosing [9, 10]. Though the results of numerous ongoing trials give reason to expect wider scale use of single-low dose primaquine for reducing *P. falciparum* transmission, there is growing interest in identifying novel transmission reducing compounds [7].

For screening new compounds, several assays determining gametocyte viability activity have been developed. These have been based on the expression of metabolic markers or reporter genes, or more recently on gametocyte activation in vitro measured by the presence of female and male gametes [11-13]. Such assays are, however, not always predictive of blocking transmission of the parasite to the mosquito, and agents with putative TRA are best tested in the standard membrane feeding assay (SMFA). In the SMFA cultured gametocytes are mixed with blood and test compounds, and this mix is fed to laboratory reared mosquitoes to determine the effect of the compound on the parasites establishment in the mosquito. As the outcome of the SMFA is mosquito infection and the timing and duration of compound exposure is controlled, the assay is easily adapted to assess effects active at any stage of parasite development – from gametocyte inhibition, to inhibition of sporogony [13, 14]. The SMFA has numerous pitfalls though, first among these being the need to dissect mosquitoes and count oocysts on the mosquito midgut by microscopy; a labour intensive and subjective task, which imposes significant limits on the assays scalability. We previously described the use of a parasite line expressing a fusion of the green fluorescent and firefly luciferase proteins (NF54HT-GFP-Luc) in a new SMFA format based on the measurement of luciferase activity in groups of homogenised individual or pooled mosquitoes [15]. Although very effective, this strain may be sub-optimal for drug screening; introduction of the human dihydrofolate reductase (hDHFR) selection marker during integration of the GFP-Luc expression cassette renders it resistant to antifolates

[16] and makes it necessary to maintain exposure to antifolate compound WR99210 during parasite culture to prevent reversion to wild-type (WT), possibly modifying its response to other inhibitors by affecting parasite metabolism. Here we report the development of a parasite line that constitutively expresses GFP-Luc and is free of limiting selection markers, for use in an efficient, dissection-free SMFA screen. Our approach used frozen mosquitoes and machine homogenisation in a 96-well format to improve the efficiency of indiscriminate drug screening. After validation and comparison of luciferase expression with microscopy based oocyst counts we used our luminescent SMFA to screen 47 marketed and experimental antimalarials and compared the results with data from the gametocytocidal lactate dehydrogenase (pLDH) activity assay and Pfs25 AlphaLISA assay. Furthermore, the luciferase screen was used to evaluate the TRA of 18 compounds selected from the Medicines for Malaria Venture (MMV) malaria box specifically for their potential activity as gametocytocidals. In this final screen, gametocytocidal or permanent inhibitory activity was confirmed by incubating gametocytes with compounds before 'washing out' the compound prior to mosquito feeding, while specific activity against the parasite stages emerging in the mosquito gut was confirmed by adding compounds to the gametocyte culture immediately prior to mosquito feeding. These assays identified novel transmission blocking small molecules and provide new insight into the predictive value of *in vitro* metabolic and gamete formation inhibition assays, showing they can miss potent compounds, and highlighting the advantages of the gold-standard SMFA for screening transmission blocking compounds. Importantly, our more scalable luciferase based read-out increases throughput of the assay and makes compound screening with the SMFA less resource intensive than when performed with microscopy.

Results

Generation of a GFP-Luc expressing parasite line free of selection markers

To allow unrestrained evaluation of new compounds we developed a new parasite line, free of drug selection markers (full title: NF54-ΔPf47-5'*hsp70*-GFP-Luc; hereafter: NF54-HGL), that stably expresses a GFP-Luciferase fusion protein under control of the constitutive *P. falciparum hsp70* promoter (**Figure 1 A-B**).

To confirm the proposed effect of removal of the hDHFR selection marker on antifolate sensitivity, we compared sensitivity of wildtype NF54, intermediate clone NF54-HGL-hDHFR (created prior to FLP recombinase-mediated excision of the hDHFR maker) and NF54-HGL asexual blood stage parasites to dihydroartemisinin (DHA), chloroquine, and two antifolate drugs: WR99210 and Pyrimethamine. The half maximal inhibitory concentration (IC50) for DHA and chloroquine are comparable for all three parasite lines.

As predicted, presence of the hDHFR selection marker in NF54-HGL-hDHFR renders this parasite resistant to WR99210 and Pyrimethamine. Excision of the hDHFR open reading frame effectively restores sensitivity to these antifolates for NF54-HGL (**Figure 1 D-G**). Since pLDH is a stable enzyme and antifolates have relatively slow parasite killing kinetics, the inhibition by WR99210 and pyrimethamine did not reach 100% within the timeframe of the assay [17].

Assessment of GFP-Luc expression

Analyses of luminescence signals confirmed expression of the GFP-Luc protein in asexual blood stage parasites (**Figure 1 C**). In line with previous reports on different expression levels in the various life cycle stages of the parasite [18], expression was higher in day 8 gametocytes than in mature gametocytes and asexual blood stages. To assess GFP-Luc expression during the parasites development in mosquitoes, *Anopheles stephensi* were fed a blood-meal containing NF54-HGL gametocytes and dissected at various time-points post infection (PI) to allow visual inspection of the mosquito midgut. No fluorescence was observed during ookinete formation (24 h PI, data not shown), indicating that GFP expression levels may have been under the limit for microscopical detection. From day 6 PI GFP positive oocysts were easily observable **(Supplementary figure S1**). Luciferase expression was weak during early sporogonic development (2-6 days PI, data not shown), but robust signal in low intensity infections (mean oocysts = 1.28, range = 0-7) was detectable from day 7 PI onwards (**Supplementary figure S2**). Uninfected negative control mosquitoes showed low levels of luminescence (n=617; mean RLU=3.2 RLU; standard deviation=1.9 RLU). Day 8 PI showed a clear separation of positive and negative mosquitoes and was chosen for subsequent assessments of infection intensity (*i.e.* number of oocysts per midgut) and prevalence (*i.e.* percentage of mosquitoes with oocyst stage infection) based on luminescence intensity.

Microscopy and luciferase based assessments of infection and TRA

To confirm that measurements of luciferase activity can replace manual dissection-based oocyst counts, as was previously shown for NF54HT-GFP-Luc [15], 172 experimental mosquito feeds (hereafter 'feeds') were performed using NF54-HGL gametocyte culture. From each feed, separate mosquito samples were assayed by standard microscopy and by luminescent SMFA (microscopy, n=2557; luminescence, n=3570). Thirty-two of these feeds were performed as controls allowing for direct comparison of infection intensity and prevalence based on oocyst and luminescence readouts. These were performed either without test compounds (0.1% DMSO, n=16) or with control compound DHA that shows gametocytocidal activity at high concentration (10 μM DHA, n=16) [13]. Mean oocyst counts for control feeds were between zero and 29 oocysts (mean 4.9 oocysts), and had a positive linear relationship with mean luminescence intensity (R^2=0.86, $p<0.0001$).

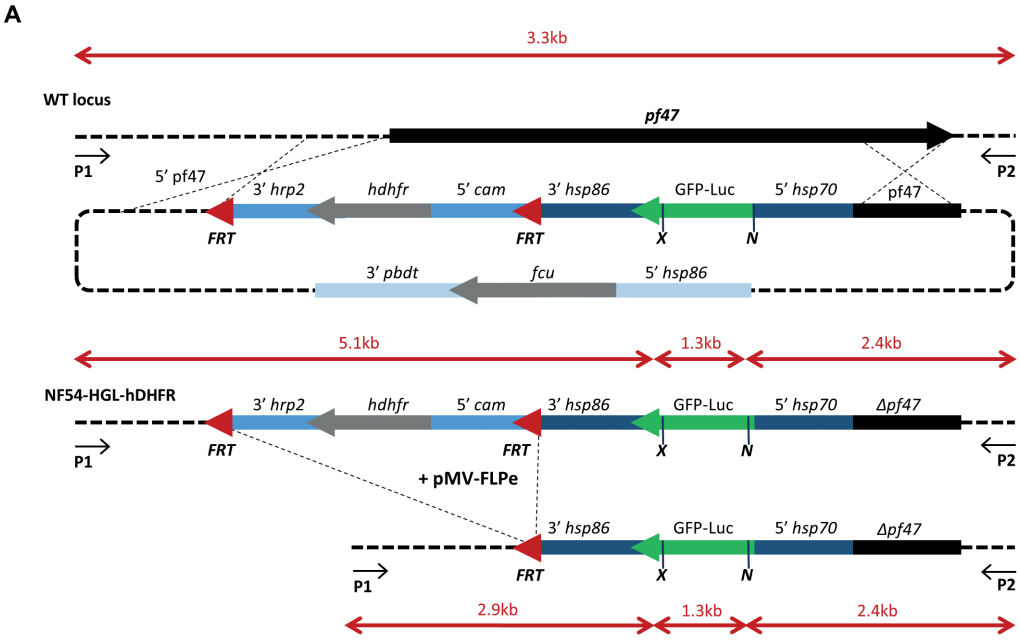
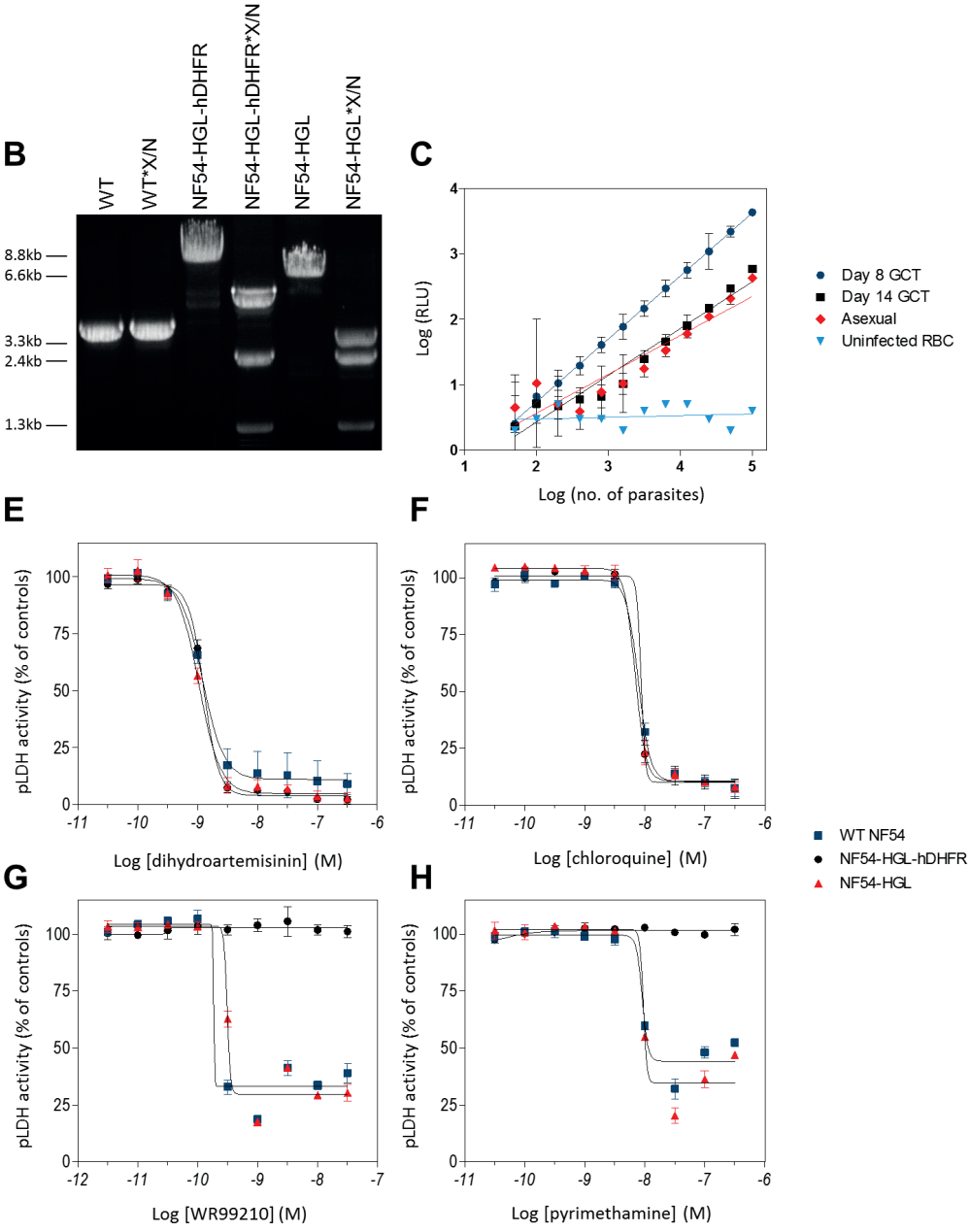
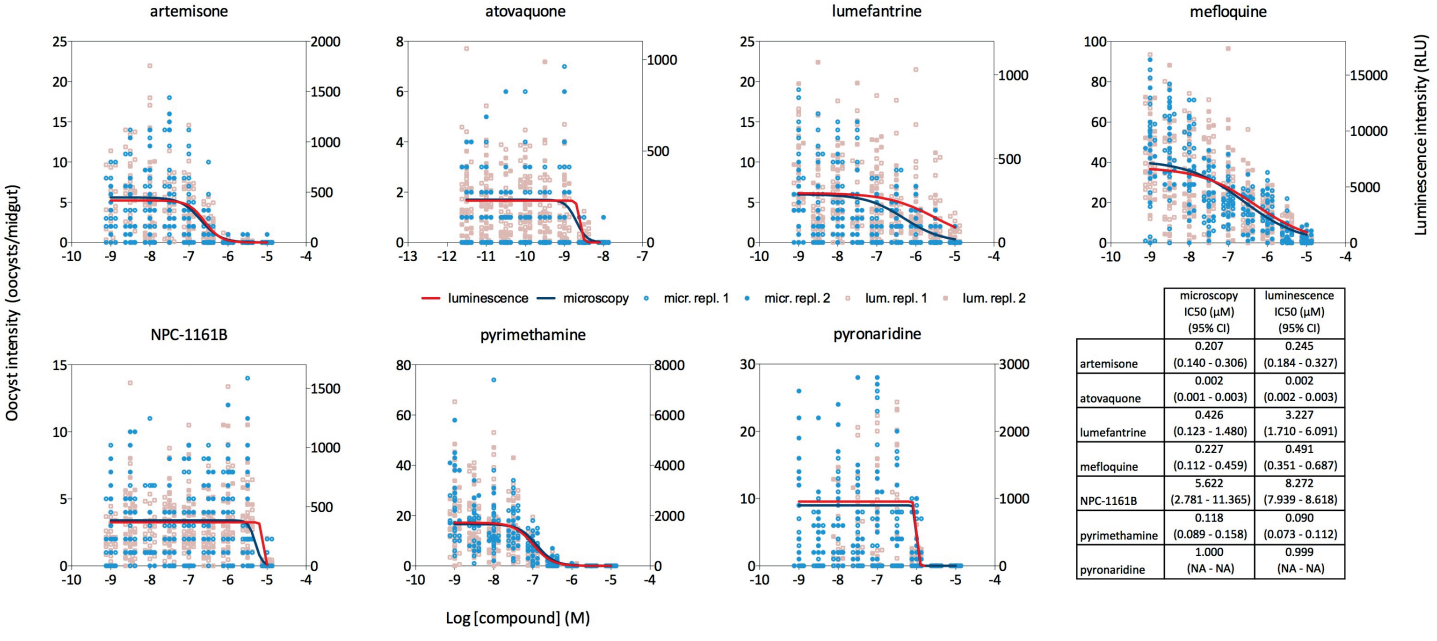


Figure 1. A. Schematic of the gene targeting strategy to insert a GFP-Luc reporter gene under control of the *hsp70* promoter in the *pfs47* locus, showing the genomic locus of the wild-type (WT) and gene deletion mutants Δpf47 containing the GFP-luciferase ORF before and after removal of the *hdhfr* resistance marker. *P1, P2*: primers MWV300 and MWV301, respectively, used for PCR analysis; *X* (*XcmI*), *N* (*NcoI*): restriction sites used for conformation of LR-PCR analysis; *pf47*: *pf47* locus; *hrp:* histidine rich protein; *cam:* calmodulin; *hsp:* heat shock protein; *hdhfr*: human dihydrodrofolate reductase coding region; *GFP-Luc*: GFP-luciferase fusion protein; *fcu:* cytosine deaminase/uracil phosphoribosyl transferase; *pbdt: P. berghei dhfr* transcription termination region; kb: kilo basepairs. **B.** Verification of the integration site by PCR and restriction digestion. Genomic DNA fragments from the parental NF54 strain (WT), intermediate transgene NF54-HGL-hDHFR and final transgene NF54-HGL were amplified by PCR using primers MWV300 and MWV301 (P1 and P2) and analysed by DNA gel electrophoresis. Where indicated, the PCR fragment was subjected to restriction digestion with *XcmI/NcoI* (X/N) **C.** Luminescence intensity (relative light units, RLU) as a function of cell number. The figure shows a comparison between uninfected red blood cells (RBC), immature gametocytes (Day 8 GCT), mature gametocytes (Day 14 GCT) and asexual blood stage parasites. **D-G.** FLP-FRT mediated excision of the selection marker restores sensitivity to antifolates. Drug sensitivity analysis of WT, ΔPf47GFP-luc-hdhfr and ΔPf47GFP-luc to DHA (**D**), Chloroquine (**E**), Pyrimethamine (**F**), and WR99210 (**G**) in a dose dependent asexual growth inhibition assay using pLDH enzyme activity as a readout. Depicted data represents a triplicate measurement and was normalized to the MIN (10 μM DHA) and MAX (0.1% DMSO) controls. Error bars indicate standard deviations.


B
WT
WT*X/N
NF54-HGL-hDHFR
NF54-HGL-hDHFR*X/N
NF54-HGL
NF54-HGL*X/N
8.8kb
6.6kb
3.3kb
2.4kb
1.3kb
C
Log (RLU)
Log (no. of parasites)
Day 8 GCT
Day 14 GCT
Asexual
Uninfected RBC
E
pLDH activity (% of controls)
Log [dihydroartemisinin] (M)
F
Log [chloroquine] (M)
G
Log [WR99210] (M)
H
Log [pyrimethamine] (M)
WT NF54
NF54-HGL-hDHFR
NF54-HGL



	microscopy IC50 (µM) (95% CI)	luminescence IC50 (µM) (95% CI)
artemisone	0.207 (0.140 - 0.306)	0.245 (0.184 - 0.327)
atovaquone	0.002 (0.001 - 0.003)	0.002 (0.002 - 0.003)
lumefantrine	0.426 (0.123 - 1.480)	3.227 (1.710 - 6.091)
mefloquine	0.227 (0.112 - 0.459)	0.491 (0.351 - 0.687)
NPC-1161B	5.622 (2.781 - 11.365)	8.272 (7.939 - 8.618)
pyrimethamine	0.118 (0.089 - 0.158)	0.090 (0.073 - 0.112)
pyronaridine	1.000 (NA - NA)	0.999 (NA - NA)

Figure 2. Standard Membrane Feeding Experiments for 7 compounds from the MMV validation box, assessed both by microscopy and luciferase assay. All compounds were tested in nine dilutions in duplicate. The figure shows oocyst counts in individual mosquitoes and relative light units (RLU) from a distinct cohort of mosquitoes from the same cage. The table shows IC50 values and cognate 95% confidence intervals (CI) determined from fitting the data to a logistic regression model by Maximum Likelihood Estimation. NA: confidence interval not computed as the model did not converge.

Similarly, oocyst prevalence was positively associated with luminescence based prevalence (R^2=0.95, p<0.0001) (**Supplementary figure S3**). Combining all mosquitoes from control feeds, prevalence estimates for all mosquitoes from control feeds were 43.33% (208/480) for microscopy, and 49.22% (350/711) for luminescence.

To further validate the luminescent SMFA we tested seven compounds from the MMV validation box in full dose response in an 'indirect' luminescent SMFA format. In the indirect assay, gametocytes were incubated with compounds for 24 hours, and mosquitoes were fed a blood-meal containing this gametocyte culture/compound mix. Microscopic examination of oocyst densities in midguts and luminescence measurements were performed on distinct cohorts of mosquitoes from the same cage. As shown in **figure 2**, all compounds dose-dependently reduced oocyst formation and luminescence intensity. Data were fitted to a Hill equation using Maximum Likelihood Estimation to find the best fit. The dose response curves fitted on the microscopy and luminescence data aligned very well for artemisone, atovaquone, mefloquine, NPC1161B, pyrimethamine and pyronaridine. The curves for lumefantrine appeared to be less well aligned due to a distinct dispersion of oocyst counts and RLU values at the highest concentration tested. For all other compounds the derived IC50 values differ by no more than 2-3 fold when comparing the two read-outs.

The luminescent SMFA identifies compounds with different modes of TRA

Using only luminescence readouts, we screened 47 established antimalarial compounds in the indirect luminescent SMFA format at a 5 μM concentration. Samples of 22-24 mosquitoes were analysed for each compound (n=46*24; 1*22). The results of this rapid screen are shown in **Figure 3A**, with TRA (%) calculated using generalised linear mixed models (GLMM) from differences in the intensity of infection between test and vehicle (0.1% DMSO) control feeds. Thirty-four compounds had statistically significant TRA based on reductions in luminescence intensity; 21/34 with ≥80% TRA [15].

To provide more information on the mechanism of active compounds, all compounds showing ≥80% TRA on the intensity scale in the indirect assay were re-tested, this time adding compounds to the blood/gametocyte culture mix immediately prior to mosquito feeding (hereafter, the 'direct' assay variant). Fourteen compounds had >80% TRA in the indirect assay but not in the direct assay, suggesting the absence of strong activity against the parasite life stages occurring in the mosquito gut. Amino alcohol halofantrine, antifolates P218, pyrimethamine and chlorproguanil, synthetic dye methylene blue, BC1 inhibitor atovaquone, and protein synthesis inhibitor cycloheximide had comparable TRA in the indirect and direct SMFA formats, indicating activity against mosquito stage parasites, but not excluding the possibility of concurrent gametocytocidal action.

The results of the indirect and direct assays were compared with previously described data [13] showing the effect of the same 47 compounds at 5 μM in a gametocyte viability assay with pLDH readout, and a homogeneous immunoassay detecting the Pfs25 antigen

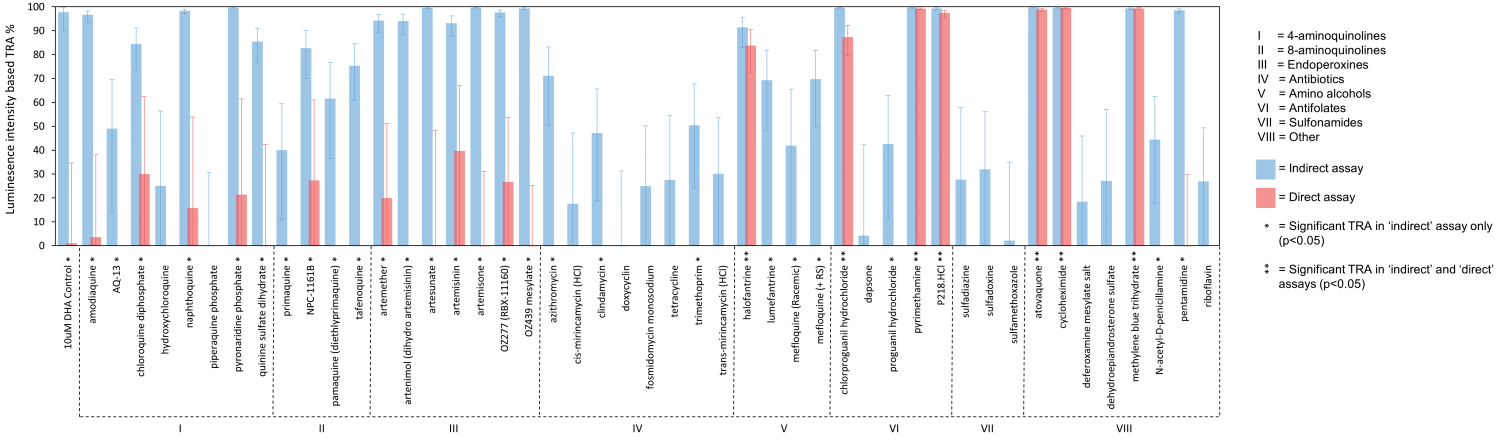


Figure 3. A. Single concentration (5 µM) rapid luminescent SMFA screen of 47 compound from MMV validation box, assessed with the indirect and direct assay variants. TRA% was calculated from relative differences between test and control feed luminescence intensity. Error bars indicate 95% confidence intervals. **B.** Heat map comparing the SMFA data to published data from pLDH and Pfs25-based gametocyte viability assays. The numbers indicate percentage inhibition at a compound concentration of 5 µM. A color scale from green to red indicates low to high TRA.

	pLDH	Pfs25	indirect SMFA	direct SMFA
4-aminoquinolines				
Amodiaquine	9	94	97	4
AQ-13	61	25	49	NA
Chloroquine diphosphate	70	63	84	30
Hydroxychloroquine	12	7	25	NA
Naphthoquine	95	-4	98	16
Piperaquine phosphate	22	15	-11	NA
Pyronaridine phosphate	61	101	100	21
Quinine sulfate dihydrate	23	29	85	-12
8-aminoquinolines				
NPC-1161B	16	42	83	27
Pamaquine (diethlyprimaquine)	21	-8	62	NA
Primaquine	8	-3	40	NA
tafenoquine	-2	30	75	NA
amino alcohols				
Halofantrine	26	19	91	84
Lumefantrine	19	17	69	NA
Mefloquine (+ RS)	39	39	42	NA
Mefloquine (Racemic)	41	43	70	NA
antibiotics				
Azithromycin	47	33	71	NA
Cis-Mirincamycin (HCl)	4	6	18	NA
Clindamycin	6	1	47	NA
Doxycyclin	-2	-3	-13	NA
Fosmidomycin monosodium	9	-20	25	NA
Tetracycline	-6	10	28	NA
Trans-Mirincamycin (HCl)	18	9	30	NA
Trimethoprim	-4	2	50	NA

	pLDH	Pfs25	indirect SMFA	direct SMFA
antifolates				
Chlorproguanil hydrochloride	97	84	100	87
Dapsone	9	-6	4	NA
P218.HCl	0	-2	99	97
Proguanil hydrochloride	32	17	43	NA
pyrimethamine	4	2	100	99
endoperoxides				
Artemether	69	98	94	20
Artemisinin	45	91	93	40
Artemisone	75	98	100	-22
Artenimol (Dihydro artemisinin)	86	85	94	NA
Artesunate	88	100	100	-5
OZ277 (RBX-11160)	79	98	98	27
OZ439 mesylate	33	91	99	-41
sulfonamides				
Sulfadiazine	0	-11	28	NA
Sulfadoxine	-2	-4	32	NA
Sulfamethoxazole	9	-4	2	NA
other				
Atovaquone	5	29	100	99
Cycloheximide	26	92	100	100
Deferoxamine mesylate salt	4	-14	18	NA
Dehydroepiandrosterone sulfate	-3	-4	27	NA
Methylene Blue trihydrate	99	95	99	99
N-acetyl-D-penicillamine	0	-14	44	NA
Pentamidine	88	99	99	-7
Riboflavin	17	13	27	NA

B

The luminescent SMFA identifies compounds with TRA that are missed by gametocyte and gametogenesis assays

on emerging female gametes (**Figure 3B**). At a threshold of ≥80% inhibition, the pLDH and gametogenesis assays identified 6/47 and 13/47 compounds respectively as having gametocytocidal activity. The activity of all but one of the six compounds identified by the pLDH assay (napthoquine) was confirmed by the gametogenesis assay, while all hits from either viability assay were confirmed in the indirect luminescent SMFA. However, high TRA was observed for a number of compounds where the inhibition assays showed low activity, indicating that some compounds had activity only identifiable with transmission as the definitive readout. Total false negative rates for the pLDH and gametogenesis assays were 32% (15/47) and 17% (8/47), respectively. Excluding compounds that blocked transmission in the direct luminescent SMFA, which we presumed to have activity downstream of the gametocyte, the SMFA still identified a larger number of transmission-blocking compounds than either gametocytocidal assay, with false negativity rates of 25% (10/40) in the pLDH assay and 10% (4/40) in the gametogenesis assay. This is not surprising since the SMFA covers a higher biological content than either of the other two methodologies.

Identification of novel transmission-blocking small molecules

In recent years, a number of gametocyte assays have been developed that attempt to predict gametocyte viability and infectivity based on metabolic activity, reporter gene expression, or changes in cell shape or antigen expression [11-13, 19-26]. In an effort coordinated by the Bill & Melinda Gates Foundation and the Medicines for Malaria Venture (MMV), a number of these and additional novel assays selected a subset of compounds from the MMV Malaria box as potentially transmission blocking. All of these molecules kill asexual blood stage parasites with submicromolar IC50s (**Table 1**). Here, we evaluated 18 of these selected compounds in full dose-response experiments using the luminescent SMFA. In order to confirm a gametocyte-based mechanism of action, compounds were washed out following incubation with stage V gametocytes to prevent activity against parasite stages emerging in the mosquito midgut. Effectiveness of the washing step was tested by evaluating the activity of pyrimethamine, which has potent activity against the parasite stages that develop in the mosquito midgut (**Figure 2**) but not against gametocytes [27]. Indeed, washing a culture of gametocytes exposed to pyrimethamine restored subsequent oocyst development in the mosquito, confirming that this format of the assay is likely restricted to the analyses of gametocytocidal modes of actions (**Supplementary figure S4**). This might however be different for more lipophilic drugs, which might be washed out with less efficiency. Sixteen out of the 18 Malaria box compounds tested in this wash-out mode of the SMFA dose-dependently reduced oocyst development in the mosquito midgut (**Figure 4**). Compounds MMV665941, MMV667491, MMV019918 and MMV665827 were among the most potent transmission-reducing molecules, with IC50 values of 0.04, 0.06, 0.07 and 0.1 μM, respectively. To reveal whether these compounds act against the parasite stages that develop in the mosquito midgut on top of their gametocytocidal activity, all compounds were tested

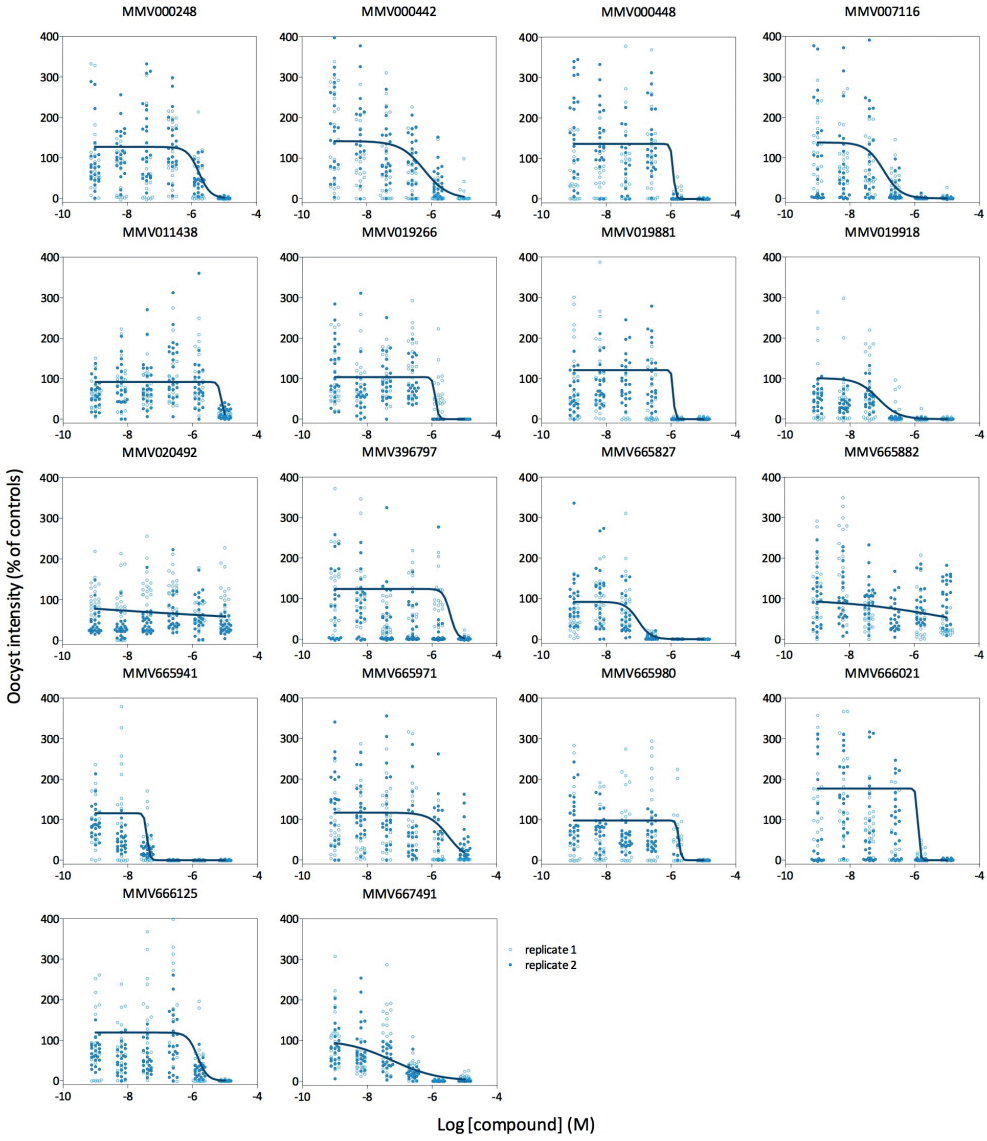


Figure 4: Dose response experiments for 18 compounds from the MMV malaria box, assessed in the luminescent SMFA in a wash-out assay variant. All compounds were tested in duplicate in two independent experiments. The figure shows oocyst intensities relative to vehicle control (0.1% DMSO) as a function of concentration for the compounds indicated at the top of the panels. The compounds depicted in their molecular structure showed an IC50 < 100 nM in the luminescent SMFA.

Table 1. Compound activity as determined in the luminescent SMFA compared to activities reported by published gametocyte viability assays and asexual replication assays

Compound	Washout SMFA		Direct SMFA	Published gametocyte assay data					Asexual stage†
	IC50 µM (95% CI)	slope (95% CI)	% inh. 10 µM	Exflagellation[12] (imaging) IC50 µM (% inh. 1 µM)	Pfs25[12] IC50 µM (% inh. 1 µM)	alamarBlue[24] IC50 µM (% inh. 5 µM)	Imaging[25] IC50 µM (% inh. 5 µM)	Exflagellation[26] (SYBR green) IC50 µM (% inh. 10 µM)	IC50 (µM)
MMV000248	1.64 (1.26 – 2.13)	-2.44 (-3.07 – -1.81)	48.76*	NA (-66%)	NA (8%)	0.31	1.1	NA (95%)	0.72
MMV000442	0.59 (.35 – 1.01)	-1.18 (-1.58 – -0.78)	5.5	NA (-12%)	NA (6%)	NA (72%)	0.92 (55%)	NA (59%)	0.36
MMV000448	1.18 (1.07– 1.31)	-11.63 (-15.91 – -7.34)	97.93*	0.25	4.02	1	0.71	5.4	0.23
MMV007116	0.11 (.06 – .19)	-1.62 (-2.15 – -1.08)	99.22*	20.75	NA (10%)	NA (40%)	0.26	NA (67%)	0.35
MMV011438	8.58 (7.95 – 9.26)	-8.47 (-12.62 – -4.32)	-3.29	NA (-77%)	NA (-31%)	0.82	1.13	NA (69%)	0.32
MMV019266	1.29 (1.21 – 1.38)	-9.38 (-12.41 – -6.35)	7.07	NA (31%)	NA (-11%)	0.31	0.33	NA (81%)	0.62
MMV019881	1.17 (. – .)	-15.81 (. – .)	88.15*	NA (57%)	NA (-7%)	NA (76%)	NA (32%)	5.5	0.65
MMV019918	0.07 (.04 – .13)	-1.36 (-1.80 – -0.93)	88.55*	0.07	0.52	0.32	0.7	0.9	0.79
MMV020492	ND	ND	-16.63	NA (36%)	NA (-12%)	NA (70%)	NA (-5%)	NA (59%)	0.03
MMV396797	3.62 (.87 – 15.18)	-4.10 (-9.87 – 1.67)	-16.44	NA (-14%)	NA (-17%)	NA (90%)	0.01	8.8	0.48
MMV665827	0.10 (.06 –.15)	-2.23 (-3.05 – -1.42)	98.25*	16.55	NA (-15%)	NA (41%)	0.34	NA (66%)	0.12
MMV665882	ND	ND	4.92	NA (-12%)	NA (-48%)	NA (92%)	0.07	NA (75%)	0.47
MMV665941	0.04 (. – .)	- 10.29 (. – .)	99.84*	0.42	0.3	NA (86%)	0.32	1.8	0.26
MMV665971	3.22 (1.45 – 7.15)	- 1.50 (- 2.57 – - 0.44)	-7.04	NA (– 29%)	NA (– 13%)	NA (73%)	0.23	NA (61%)	0.49
MMV665980	1.76 (1.57 – 1.96)	- 11.52 (- 25.29 – 2.24)	64.17*	8.07	NA (74%)	NA (27%)	NA (91%)	NA (76%)	0.21
MMV666021	1.25 (1.17 – 1.34)	- 13.30 (- 17.32 – - 9.28)	94.02*	NA (30%)	NA (– 20%)	NA (58%)	0.61	NA (68%)	0.09
MMV666125	1.40 (1.11 – 1.76)	- 2.86 (- 3.97 – - 1.76)	-24.69	NA (– 3%)	NA (– 17%)	0.48	0.75	NA (74%)	0.38
MMV667491	0.06 (.03 – .11)	- 0.61 (- 0.73 – - 0.50)	89.05*	0.09	0.18	NA (91%)	1.06	4.5	1.23

The table shows IC50 values where available (NA: not available) or the percentage inhibition at the highest concentration tested, as indicated in the header row. IC50 data for the exflagellation (imaging) and Pfs25 assays were generated in a washout format, whereas percentage inhibition values represent data from a continued exposure experiment12. For SMFA data, 95% confidence intervals are indicated between brackets where possible. For MMV019881, and MMV665941 the model did not converge. Asterisks (*) indicate statistically significant inhibition ($p < 0.05$) in the direct SMFA. † The last column shows activity against asexual blood stages of *P. falciparum* strain 3D7 as deposited at the ChEMBL repository under accession number CHEMBL2028071. ND: no dose response relationship was detected in the data.

in the direct assay variant at the same maximum concentration as in the wash-out assay (10 μM), confirming that 10/18 compounds significantly reduced mosquito oocyst intensity Compounds MMV020492 and MMV665882 that did not show gametocytocidal activity were equally inactive in the direct SMFA. To further address the mechanism of action of compounds with functional TRA, the SMFA data were compared to data from published studies that addressed different aspects of gametocyte biology (**Table 1**). MMV019918 was the only compound consistently showing activity across the different assay formats, although IC50 values ranged from 0.07 μM to 0.9 μM, with large differences between the two assays that interrogated the ability of a gametocyte to undergo exflagellation. Conversely, MMV007116 and MMV665827 showed potent activity in the SMFA but very low activity in the published gametocyte assays, with the exception of a luciferase reporter assay that revealed the effect of these compounds on gametocyte viability. In general, the luminescent SMFA identified more compounds with functional TRA than predicted by each of the individual published gametocyte assay formats.

Discussion

The firefly luciferase expressing parasite line NF54-HGL enables the SMFA to be performed for indiscriminate compound screening with a semi-automated luciferase based read-out. The results of the current study demonstrate the versatility of the SMFA for screening compounds with activity against different transmission stages of the malaria parasite, and validate the luminescent assay format in combination with microtiter plate-based processing of mosquitoes for improving the efficiency of the biological gold-standard for assessing *Plasmodium* transmission in controlled experiments.

We presented a screening pipeline for the SMFA that allowed us to investigate drug mechanism of action while retaining transmission as the assay end-point. The indirect assay, in which compounds were incubated with gametocytes for 24 hours prior to mosquito feeding, represents the simplest and most sensitive screen and the first step in the pipeline for screening large compound libraries. The direct assay, in which the compound is added at the moment the gametocyte activates to form a gamete, interrogates compound action in the mosquito midgut. Pharmacokinetics in the mosquito are largely unknown, but detoxifying enzymes may limit the compound's action in the midgut, restricting the direct SMFA to monitoring effects on gametogenesis, ookinete formation and perhaps early sporogony [28]. Lastly, the wash-out format of the SMFA addresses a mode of action that is likely restricted to the gametocyte. High-throughput screening is impossible with the SMFA, but the luminescent SMFA represents a significant improvement in the techniques scalability. The replacement of direct oocyst counts with luciferase measurements from homogenised mosquitoes in the SMFA was described previously [15] for parasite line NF54HT-GFP-luc

[16], but aspects of the methodology described here differ. The current reporter parasite is devoid of the DHFR selection marker, allowing for precise description of the effects of antifolates including pyrimethamine on oocyst development, and expediting the evaluation of a wide variety of compound libraries. Mosquito sample sizes were limited to increase throughput, and freezing mosquitoes prior to further processing increased flexibility of laboratory operations. Furthermore, semi-automated processing of mosquitoes significantly reduced the time needed for assessment of infection status. In all, the luminescent format for individual mosquitoes reduces hands-on time 2-fold in comparison with the microscopical readout, which is a tremendous step up from a previously published 'industrialized' SMFA format [29]. For even further increased throughput, screening in the luminescent SMFA may be performed by pooling larger numbers of mosquitoes, achieving an approximate four-fold increase in throughput over the standard microscopical readout if only infection intensity is the desired output [15].

Our data from a large number of infections indicate that oocyst intensity is a simple linear function of luminescence intensity. This is in keeping with our previous work that showed a linear relationship between oocyst counts and luminescence intensity, using a different promoter to drive expression of the reporter gene [15]. Oocysts are an intermediate parasite life-stage which even when developing concurrently in a host mosquito may differ in size and sporozoite content [30-32]. Whereas microscopy produces discrete data, luminescence measurements provide continuous data that reflect actual numbers of developing sporozoites, which may be more directly relatable to mosquito infectivity, for which oocyst counts are an accepted proxy. An added advantage of the luminescent assay is that it is less subjective than microscopy based assessments of oocyst numbers. We cannot exclude the possibility that in heavily infected mosquitoes, with higher oocyst intensities than those reported here, nutrient and space competition may lead to lower *per capita* sporozoite production, resulting in a non-linear relationship between luminescence and oocyst intensity and lower luminescence based TRA estimates than would be determined from oocyst number alone. Though we advise caution in extrapolating actual oocyst number from luminescence values, we believe luminescence based TRA estimates may be more accurate than those made using oocyst data.

In the direct mode of the SMFA the compound remains present in the bloodmeal, and there is a remote possibility that it interferes with activity of the luciferase reporter rather than with oocyst development. We have been unable to investigate the potential effect of compounds interacting with *hsp70* expression, as has been observed in other eukaryotes [33], or the possibility that compounds may directly inhibit luciferase protein after its production [34]. We consider both events highly unlikely to affect the luciferase readout in the current SMFA format, as compounds are at latest added to the mosquito bloodmeal 8 days before luminescence measurements. Furthermore, analyses of a subset of the MMV malaria box compounds in a luciferase inhibition assay did not reveal inhibitory activity at the

concentrations that reduce oocyst development [22]. The possibility that some compounds may effect mosquito metabolism, and by proxy parasite developmental success, can also not be excluded. Readouts based on oocyst number may be insensitive to such subtle effects, while the more quantitative luminescence readout may not be. Additional experiments will be required to elucidate the interaction of mosquito and parasite fitness in relation to compound exposure.

Apart from oocyst intensity, oocyst prevalence (the proportion of mosquitoes that develop an oocyst-stage infection), is an important parameter determining malaria transmission. Whereas inter-experimental variation in control mosquito oocyst burden has no effect on TRA expressed as a reduction in oocyst intensity, it heavily influences the outcome based on a reduction in oocyst prevalence.[35]. Since our experiments comprised a large set of experimental infections that each showed a distinct distribution of oocyst densities we have, therefore, limited our analyses to a comparison of compound effects on oocyst intensity, as this is the only parameter that is directly comparable between experiments. Nevertheless, a complete assessment of the transmission blocking potential of an antimalarial would require a description of the effects on infection prevalence, preferable at infection intensities that mimic those observed in naturally infected mosquitoes [36]. An experimental and computational strategy to achieve such an assessment has been implemented in our laboratory and will be described elsewhere.

Compounds reducing malaria parasite transmission to mosquitoes may kill gametocytes directly (gametocytocidal activity), irreversibly impair the parasites ability to develop once ingested by the mosquito, or else inhibit gametocyte developmental viability only while drug pressure is maintained in the mosquito environment [12]. Because mature gametocytes persist at low densities for several weeks after ACT treatment [6], and because submicroscopic gametocyte densities may be highly transmissible [37, 38], efforts to develop transmission-reducing compounds are focused on identifying activity against mature gametocytes [39]. *In vitro* assays based on markers of metabolic activity (pLDH [13, 19], Alamar Blue reduction [20]) or reporter genes [21, 22, 40] can be used to assess the effects of gametocytocidal compounds but may overlook compounds that manifest their action downstream of the gametocyte. Gametocyte infectivity may be assessed in greater detail with gamete formation assays, which can include gametocyte washing steps to discriminate between reversible and irreversible compound activity [12, 13, 23]. *In vitro* ookinete formation assays can also be used to identify compounds whose presence in the mosquito blood meal inhibits parasite fertilisation [41], but these assays cannot exclude the possibility of compounds reducing ookinete viability or oocyst formation. Assays measuring the viability or development of gametocytes without mosquito feeding are comparatively inexpensive and highly scalable, so will continue to be required as a prioritization step in the screening pipeline for transmission blocking drugs. The data presented here indicate, however, that increased throughput of *in vitro* gametocyte assays goes at the cost of a

considerable number of false negatives. Importantly, as no *in vitro* assay or combination of assays is able to fully interrogate the effect of compounds on all parasite developmental stages exposed to test compounds, our results underline that the SMFA remains the gold standard assessment of gametocyte infectivity. Our indirect screen of a varied set of 47 antimalarials identified compounds preventing mosquito infection by mechanisms targeting any stage of the parasites sexual development or early sporogony. Assays based on parasite metabolic processes (pLDH) and on the expression of female gamete upregulated protein Pfs25 cumulatively failed to identify seven compounds which, incubated with cultured gametocytes (NF54-HGL) in the same concentration, caused marked reductions in oocyst stage productivity as assessed by the luminescent SMFA. Though we anticipated identifying compounds with additional activity downstream of gamete formation in the SMFA, the results of the direct assay indicate that the pLDH and gamete formation assays failed to identify compounds with effects restricted to the gametocyte; three of the seven false negatives had no activity on mosquito stage parasites in the direct SMFA format. The effect of the remaining four compounds may rely on a sporontocidal rather than a gametocytocidal activity. In keeping with previous reports of their sporontocidal activity (reviewed in Butcher [42]), pyrimethamine and atovaquone blocked oocyst development but were not active in the pLDH and gamete formation assays. Atovaquone has demonstrated a long-lasting transmission-blocking effect in *ex vivo* studies with sera from drug-treated volunteers [43], but the effect on transmission in malaria endemic settings has not been described in detail. The sporontocidal effect of pyrimethamine most likely represents a class effect of dihydrofolate reductase (DHFR) inhibitors, as it was also observed for P218 and chlorproguanil. Proguanil was understandably not active in the SMFA as only its active metabolite cycloguanil binds to DHFR [44].

Analyses of eighteen compounds from the MMV malaria box revealed sixteen compounds with functional TRA that were not necessarily identified by individual gametocyte based assay formats [12, 24-26]. These 18 compounds were flagged as potential transmission blockers by a joint effort coordinated by the Bill and Melinda Gates Foundation and the Medicines for Malaria Venture under the 'Gametocyte Assays for *Plasmodium falciparum'* program. Under this program, five consortia consisting of two to five malaria laboratories each screened the MMV malaria box in a variety of gametocyte-based assays. All consortia proposed a shortlist of active molecules for further evaluation in the SMFA. The final selection of 18 molecules, governed by MMV, was then based on potency, diversity in chemical scaffolds and presumed tractability. As these compounds were expected to act at the level of the gametocyte, we applied a wash-out format to restrict the analysis to the sexual blood stage of the parasite when testing these compounds in the luminescent SMFA. For the 18 compounds subjected to this more rigorous investigation, 16 were able to reduce onward oocyst development in the mosquito with potencies ranging from 8.6 to 0.04 μM. The luminescent SMFA did not reveal TRA for MMV020492, which is in line with its lack

of activity in published gametocyte assays. Equally inactive in the SMFA, MMV020492 showed a lack of activity in most published gametocyte assays, with the exception of a luciferase assay that indicated an IC50 of 62 nM. Overall, from a qualitative perspective the luciferase assay showed the best agreement with the SMFA, as it identified 14 out of the 16 compounds with functional TRA. Assays that interrogate the ability of a gametocyte to form a male or female gamete did not identify MMV000442 as a potential transmission reducing compound [12], whereas it showed an IC50 of 590 nM in the SMFA. This indicates that gametocyte infectivity is defined by a greater set of functions than described by its sole ability to form a gamete. Quantitatively, neither of the published gametocyte assays correlated particularly well with the SMFA, as IC50 values could vary up to 180-fold (*e.g.* MMV007116 SMFA vs gametocyte exflagellation). In addition, the rank order of compounds appeared to be different for all assays. This implies that *in vitro* gametocyte assays provide limited guidance for studies aimed at an understanding of dose in relationship to efficacy. Although mouse models have been proposed to determine the efficacious dose for blockage of malaria transmission, these are restricted to *P. berghei* parasites and have limited value for predicting efficacy on human malaria. In addition, *P. berghei* infection intensities are much higher than *P. falciparum* infection intensities, which leads to an underestimation of TRA when estimated from differences in infection prevalence [35, 45].

Our studies identified four compounds from the malaria box with IC50 values below 100 nM in the luminescent SMFA. Compounds MMV665827, MMV665941, and MMV667491 lack structural properties that meet Lipinski's rule of five that predicts oral absorption of a candidate drug [46]. Therefore, these compounds are considered tool compounds rather than drug-like molecules [47]. Nevertheless, these compounds may have value in the identification of mechanisms underlying transmission-blocking activity, which remain poorly understood. Importantly, these molecules have activity against asexual blood stage parasites, which opens the possibility of selecting drug resistant mutants followed by whole genome sequencing. This strategy has been successfully employed in the identification of PI4K, and eEF2 and confirmation of DHODH as bonafide drug targets [48-50]. From the group of compounds with potent transmission blocking activity, MMV19918 appears to qualify as a drug-like molecule and may, besides serving as a tool in unravelling mechanism of action, act as a starting point for further optimization. This compound appeared 10-fold more active in the SMFA as in assays interrogating activity against the asexual blood stages. Such a transmission-selective profile was also observed for MMV665941 and MMV667491. To the best of our knowledge these are the first molecules described to date that show selectivity towards the transmission stages of the parasite. Recently, the malaria drug development pipeline was enriched with molecules like OZ439, DSM265 and DDD107498, which unlike the majority of marketed antimalarials have activity against the transmission stages of the parasite[51]. However, for all these molecules this activity is at par or less potent than the activity observed against asexual blood stage parasites [13, 49, 50, 52]. The

observation of a transmission-selective profile in the study presented here suggests a novel mechanism of action outside the range of targets described to date. In view of the need for molecules that address a target candidate profile for prevention of transmission [53], these mechanisms and the molecules identified here warrant further investigation.

Conclusion

The results of the current study demonstrate the sensitivity and flexibility of the SMFA for determining the effect of small molecules on *P. falciparum* gametocyte transmission to mosquitoes, while validating a method that increases the assays scalability and ease of use. Comparison to gametocyte inhibition assays indicates that parasite development in the mosquito may be required to ensure assay sensitivity to transmission-blocking small molecules. Of the 18 compounds selected in this analysis based on the results of previous gametocyte assays, our data indicate that 3 compounds have greater activity on transmission stage parasites than asexual stage parasites. These molecules may be of particular interest for further drug discovery and elucidation of novel drug targets.

Methods

Parasites and culture

Asexual blood stages of all *P. falciparum* parasite lines were cultured in RPMI 1640 medium supplemented with 367 μM hypoxanthine, 25 mM HEPES, 25 mM sodium bicarbonate and 10% human type A serum, in a semi-automated system under standard conditions and induction of gametocyte production was performed as previously described [54-56].

Generation of NF54-HGL-hDHFR and NF54-HGL parasite lines

The GFP::LUC fusion protein under control of the *hsp*70 promoter was stably integrated into the genome of asexual stage NF54 (WT) parasites at the *pfs47* locus (PF13_0248) by standard double crossover recombination and positive/negative selection [57],[58, 59]. The targeting plasmid was based on the gene deletion construct pHHT-FRT-Pf5236 described previously [60]. The *p52* 3' target region in pHHT-FRT-Pf5236 was replaced by the 3' *pfs47* target region amplified by PCR using primers MWV257 and MWV259 (primer sequences in supplemental **Table S1**) and introduced in the plasmid by *MluI/SacII* restriction digestion and ligation. The 5' *p52* region was replaced with the 5'*pfs47* target region amplified by PCR using primers MWV262 and MWV267 and introduced in the plasmid by *XmaI/NarI* restriction digestion and ligation. A reporter gene cassette was constructed by subcloning a GFP::LUC fragment from plasmid PL1063 [61] in pCR2.1-BLUNTII-TOPO after *AflII/XbaI* restriction digestion. An AgeI restriction site was introduced immediately upstream of the start codon by introducing a PCR fragment amplified with primers MWV252 and

MWV253 through *KpnI/HpaI* restriction digestion and ligation. The promoter region of the *hsp70* gene (PF3D7_0818900) was placed upstream of GFP through *sacII/AgeI* restriction digestion and ligation after amplification with primers MWV198 and MWV200 and subcloning in pEGFP-N1 (Clontech laboratories, Mountain view, CA) through *BamHI/SacII* digestion and ligation. The 3' transcription termination region from the *hsp86* gene (PF3D7_0708400) was introduced following PCR amplification using primers MWV268 and MWV271 and *XbaI/ApaI* restriction digestion and ligation. Finally the reporter cassette was introduced in the gene targeting plasmid by *KpnI/NgoMIV* restriction digestion and ligation yielding plasmid pMV163.

Pfs47 target regions, 5'hsp70 and 3'hsp86 fragments were amplified by PCR amplification (Phusion, Finnzymes) from genomic *P. falciparum* DNA (NF54 strain) and all PCR fragments were sequenced after TOPO TA (Invitrogen) sub-cloning.

Transfection of wild type NF54 asexual parasites with DNA construct pMV163 was performed as described previously [59] using a Electro Cell Manipulator 600 (BTX, Holliston, USA) Double crossover mutants were selected by positive and negative selection as described [57], yielding parasite line NF54-HGL-hDHFR. Cloning of transgenic parasites was performed by limiting dilution in 96-well plates as described [62]. Parasites in positive wells were transferred to the semi-automated culture system and cultured for further phenotype and genotype analyses.

Removal of the hdhfr selection marker was next performed by transfection of the mutants with construct pMV-FLPe and selection on blasticidin [60, 63], resulting in parasite line NF54-HGL. Integrity of the transgenes was analysed by PCR using primers MWV300 and MWV301 and *XcmI/NcoI* restriction digestion.

To confirm functional FRT-mediated excision of the hDHFR selection marker, sensitivity to anti-folates and a number of non-folate reference compounds were performed on NF54 (WT), NF54-HGL-hDHFR, and NF54-HGL using a modified pLDH assay. Briefly, non-synchronised asexual parasites (~50% ring stages, 25% trophozoites and 25% schizonts) were seeded at a density of 0.5% parasitaemia in 3% haematocrit in a black 96-well plate in 50 µl of culture medium, with 50 µl of diluted test compound. Following a 72 hour incubation at 37°C, 98% humidity, 93% N2, 4% CO2 and 3% O2, the increase in parasitaemia was assessed using the modified pLDH assay as described previously [13]. Assays were performed in triplicate (three serial dilutions for each compound) and all plates included triplicate controls to assess minimum (MIN control, 1 µM DHA) and maximum (MAX control, 0.1% DMSO) signal. To compare the three different strains data were normalized and expressed as the percentage reduction in replication relative to the blocking and non-blocking controls.

GFP fluorescence analysis

The midguts of mosquitoes fed previously with NF54-HGL gametocyte culture were mounted on glass slides in a droplet of PBS, and GFP expression was visualized and photographed on a Leica fluorescence microscope with digital camera.

Standard Membrane Feeding Assays

In the 'indirect' mode of the SMFA, gametocytes were pre-incubated with test and control compounds prior to mosquito feeding. To this end, compounds were dissolved in DMSO to achieve a stock solution of 10 mM that was serially diluted in DMSO to achieve concentrations 1000-fold above the final test concentration. Subsequently, 10 µl of diluted compound in DMSO was added to 990 µl pre-warmed RPMI 1640 medium supplemented with 367 µM hypoxanthine, 25 mM HEPES and 25 mM $NaHCO_3$ ('incomplete medium'). 40 µl of this intermediate dilution was added to 360 µl of parasite culture and incubated for 24 hrs at 37°C in Eppendorf tubes, resulting in 0.1% final DMSO concentration. Hereafter, 300 µl of the gametocyte culture/compound mix was added to 180 µl of packed red blood cells and centrifuged for 20 seconds at 10,000 x *g*. After carefully aspirating the supernatant, 200 µl of human serum type A was added to the pellet. Finally, 300 µl of this mix was immediately injected into an individual membrane covered minifeeder, where 50 female *A. stephensi* mosquitoes were allowed to feed for 10 minutes. In the 'wash-out' mode of the SMFA, compounds were washed out following preincubation by adding 14 ml incomplete medium supplemented with 10% human type A serum ('complete medium') to the gametocyte suspension, followed by centrifugation (2000 x *g*) for 7 minutes. The supernatant was removed and the gametocytes were washed again with 14 ml of complete medium. The pellet was resuspended in 300 µl of complete medium and combined with 180 µl packed red blood cells. All media used for washing were prewarmed to 37°C and the subsequent preparation of the blood meal was as described above. For the 'direct' mode of the SMFA, 300 µl parasite culture was added to 180 µl red blood cells, centrifuged for 20 seconds at 10,000 g and the supernatant was carefully aspirated. Meanwhile, 10 µl of compound diluted in incomplete medium was added to 490 µl human serum type A (0.1% DMSO final). 200 µl of this human serum/compound mix was added to the gametocyte culture pellet, which was subsequently used for the SMFA as described above. For dose response analyses, compounds were diluted in DMSO with subsequent dilution in culture medium to a final DMSO concentration of 0.1%. Controls included vehicle (0.1% DMSO) and DHA at its approximate IC90 (10 µM). For all assay types, unfed and partially fed mosquitoes were removed from the cage after feeding, and fed mosquitoes were maintained at 26°C, 80% humidity. Mosquitoes were either dissected and examined for midgut oocysts on day 6-8 PI as described [64, 65], or processed in the luciferase activity assay on day 8 PI.

Mosquito processing and luciferase assay

To enhance flexibility, and to ensure that individual mosquitoes were safely and efficiently transferred from feeding cages to 96-well plates for homogenisation, mosquitoes were immobilised at day 8 PI and stored at -20 °C in sealed 15ml falcon tubes for at least 24 hours and up to 2 weeks, before being processed in the luciferase assay. Upon removal from storage at -20 °C mosquitoes were thawed and individually added to wells in shallow 96-well plates containing 60 µl PBS with Complete protease inhibitor cocktail according to the instructions of the manufacturer (Roche, Switzerland), and 0.2 gram of 1.0 mm diameter zirconia beads (Lab Services BV). Plates were next placed in a Mini-Beadbeater-96 (Biospec, Bartlesville, OK) and rocked for 2*15 seconds, with 1-2 minutes on ice between rounds of homogenisation. Based on visual inspection, this procedure achieved a homogenate consistency in line with that achievable using a hand-held pestle rotator as described previously [15]. For the individual mosquitoes 45 µl of homogenised material was transferred to wells in a Krystal 2000 black & white 96-well plate (Porvair, United Kingdom). Next 45 µl of Bright-Glo luciferase assay substrate (Promega, Madison, WI) was added to each well using a Cybi Selma dispensing station (Cybio, Germany) and the plate was incubated for 3 minutes at room temperature. Luciferase activity was measured using a Synergy 2 multi-purpose plate reader (Biotek, Winooski, VT). Background was determined by measuring uninfected mosquitoes that had undergone the same treatment.

pLDH and Pfs25 assays

Assays were performed as described previously [13].

Data analysis

Statistical analysis was conducted using R (Foundation for Statistical Computing, Vienna, Austria), STATA 12 (StataCorp., TX, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA).

All comparisons of microscopy and luciferase outcomes were conducted with separate groups of mosquitoes for each measure sampled from the same feeds. Comparisons between oocyst and luminescence based measures of mosquito infection prevalence were made with Chi-squared tests with Bonferonni correction. Associations between oocyst and luminescence intensity were quantified with linear regression. 95% confidence intervals around mean luminescence and oocyst intensity data was calculated by bootstrapping (1000 repetitions). Luminescence and microscopical TRA estimates were made using generalised linear mixed models (GLMMs) [35, 66]. For assessment of infection prevalence, the cut-off for RLU based infection positivity was determined arbitrarily as the mean luminescence intensity of all uninfected controls plus five standard deviations (12.7 RLU), as described previously [15]. For gametocyte viability and gametogenesis assays, data were expressed as

the percentage effect relative to the MIN and MAX controls [13]. For SMFAs, data were expressed relative to the negative (vehicle) controls for oocyst density and oocyst prevalence (i.e. the proportion of infected mosquitoes). IC50 values were calculated by applying a four-parameter logistic regression model using a Maximum Likelihood Estimation algorithm to find the best fit.

Acknowledgments

We want to thank Chris Janse and Shahid Kahn for providing us with plasmid PL1063. In addition, we thank Wouter Graumans, Jacqueline Kuhnen, Laura Pelser-Posthumus, Astrid Pouwelsen, and Jolanda Klaassen for mosquito breeding, and for their assistance during SMFA evaluation. Hans-Peter Duerr is gratefully acknowledged for statistical analyses, and Paul Willis for selection of compounds for SMFA evaluation. This work was supported by a grant from the Bill and Melinda Gates Foundation (OPP1040394). W.S. and T.B. are supported by PATH Malaria Vaccine Initiative and the European Community's Seventh Framework Programme (SIGNAL; Marie Curie Career Integration Grant PCIG12-GA-2012-333936).

Selected supplementary material

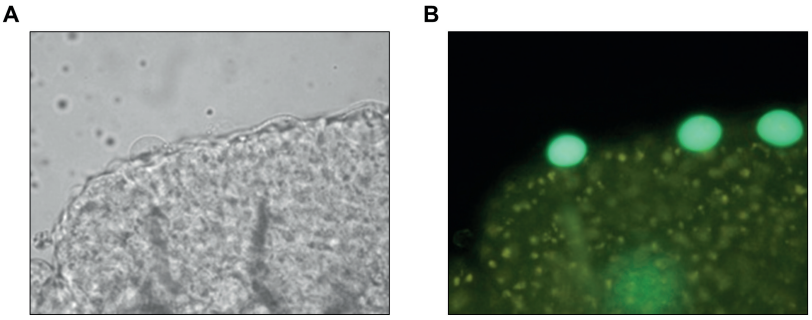
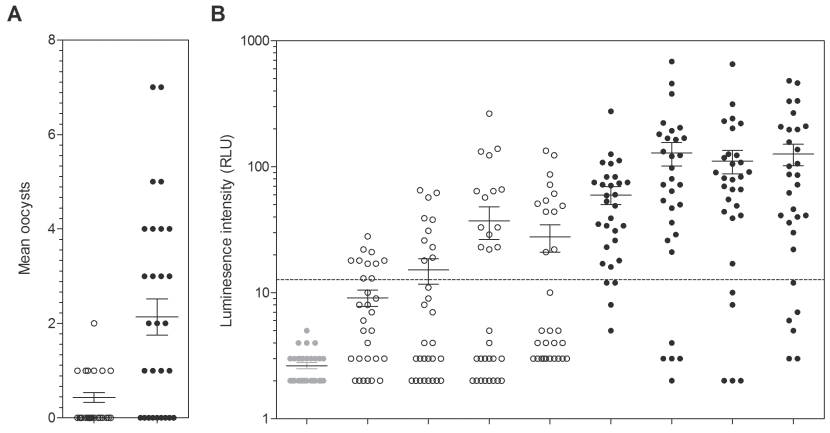


Figure S1. **A.** Brightfield micrograph of a section of mosquito midgut with 3 oocysts taken after mosquito dissection 6 days PI with NF54-HGL. **B.** Fluoresence micrograph of the same section of mosquito midgut with 3 oocysts taken after mosquito dissection 6 days PI with NF54-HGL.



Feed	A	B
Day PI	8	8
Mean oocysts	0.43	2.13
Mean RLU	-	-
Prevalence	40%	70%

Neg	A	A	A	A	B	B	B	B
-	7	8	9	12	7	8	9	12
-	-	-	-	-	-	-	-	-
2.63	9.1	15.2	37.2	27.8	59.7	128.7	111.2	126.2
-	33.3%	33.3%	46.7%	40.0%	86.7.3%	83.3%	83.3%	80.0%

Figure S2. A. Oocyst intensity in individual mosquitoes from two groups of mosquitoes (*A* and *B*) infected with NF54-HGL, determined after dissection and standard midgut staining and microscopy at day 8 PI. **B.** Luminescence intensity in individual mosquitoes from the same two mosquito groups (*A* and *B*), assessed in separate mosquito samples removed from the primary mosquito storage cage at day 7, 8, 9 and 12 PI. The dashed line indicates the cut-off calculated statistically from the RLU values of all mosquitoes assayed in the current study (8.1 RLU). Prevalence estimates were based on this cut-off.

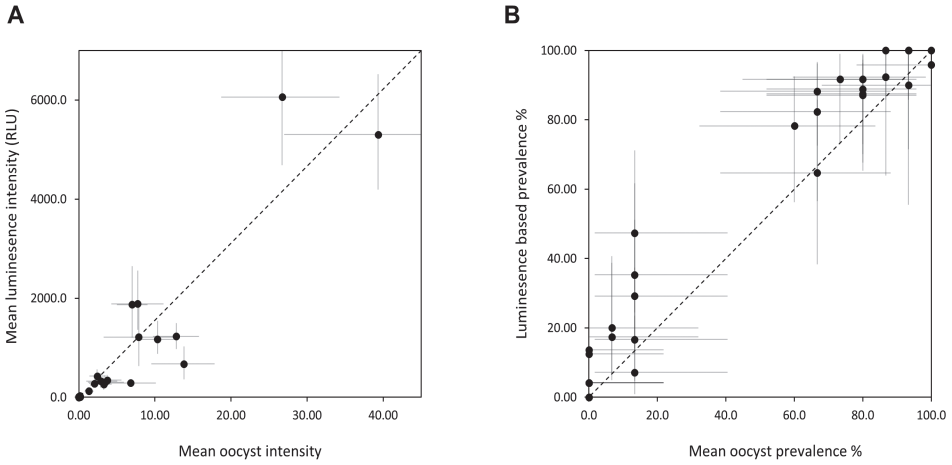


Figure S3. A. Relative oocyst and luminesence intensity among mosquitoes sampled from the same cages fed blood-meals without the addition of a test compound (16 feeds, 480 mosquitoes) or with the addition of DHA (16 feeds, 711 mosquitoes). **B.** Relative infection prevalence based on the observation of oocysts or calculated from luminesence intensity, among mosquitoes sampled from the same cages fed blood-meals without the addition of a test compound (16 feeds, 480 mosquitoes) or with the addition of DHA (16 feeds, 711 mosquitoes).

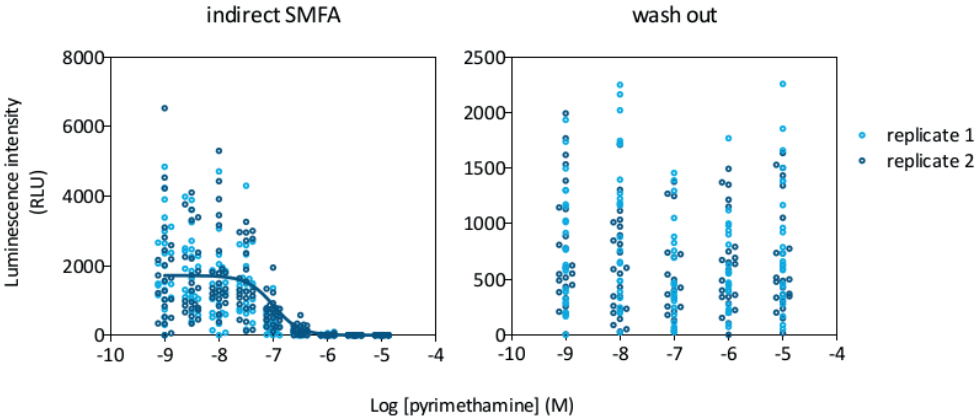


Figure S4. Efficacy of the washout protocol. The left panel shows the activity of pyrimethamine in the indirect SMFA as reported in Figure 2 of the main manuscript. The right panel shows the activity in the wash-out mode of the SMFA, where the compound was washed out prior to mosquito feeding.

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Chapter 7

The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays

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Scientific Reports. 2013; 3: 3418.

Abstract

Mosquito feeding assays are important in evaluations of malaria transmission-reducing interventions. The proportion of mosquitoes with midgut oocysts is commonly used as an outcome measure, but in natural low intensity infections the effect of oocyst non-rupture on mosquito infectivity is unclear. By identifying ruptured as well as intact oocysts, we show that in low intensity *P. falciparum* infections i) 66.7-96.7% of infected mosquitoes experienced oocyst rupture between 11-21 days post-infection, ii) oocyst rupture led invariably to sporozoite release, iii) oocyst rupture led to salivary gland infections in 97.8% of mosquitoes, and iv) 1250 (IQR 313-2400) salivary gland sporozoites were found per ruptured oocyst. These data show that infectivity can be predicted with reasonable certainty from oocyst prevalence in low intensity infections. High throughput methods for detecting infection in whole mosquitoes showed that 18s PCR but not circumsporozoite ELISA gave a reliable approximation of mosquito infection rates on day 7 post-infection.

Introduction

Malaria transmission-reducing drugs and vaccines are integral elements of the research agenda for malaria eradication [1]. Progression to a stage where these can be evaluated in clinical and community trials will be dependent on the ability of researchers to determine the infectivity of malaria exposed populations to anopheline vectors of *Plasmodium spp.* [2, 3]. This requires mosquito feeding assays (MFA), wherein groups of mosquitoes are fed the blood of potentially infective individuals and subsequently examined to confirm the establishment of infection. In preparation for trials with transmission reducing interventions (TRI), efforts are being made to standardise MFA [4-9]. Because of the laboriousness of MFAs and the necessity of large numbers of mosquito observations for precise estimates [8], increasing the scalability of MFA evaluation has been identified as a research priority [5, 6].

The scalability of MFA is to an important extent defined by the chosen endpoint. During sporogonic development, ingested male and female *Plasmodium* gametocytes quickly activate to form gametes in the mosquito gut. Ookinetes develop from the fusing of gametes, and penetrate the midgut wall forming oocysts under the basal lamina of the midgut epithelium. Upon oocyst rupture, sporozoites are released that migrate to mosquito salivary glands rendering the mosquito infective.

In the context of public health the most important measure of TRI efficacy is a reduction in the number of mosquitoes that become infective after feeding on a treated or vaccinated individual [2]. Salivary gland invasion can be detected around day 14 post infection (PI). In MFA, examining mosquitoes at an earlier time-point has advantages for mosquito husbandry, improves the likelihood of mosquito survivorship until examination, and avoids the direct health hazard that infective mosquitoes form for laboratory personnel. The presence or intensity of oocysts observed on mosquito midguts, which for *P. falciparum* can be reliably detected by microscopy as early as day 6 PI [6], is therefore commonly used as an operationally attractive outcome measure that may also be amendable for high throughput processing by immunological or molecular methods. However, this requires that the detection of any number of oocysts should reasonably predict their likelihood of causing mosquito infectivity [2]. At high oocyst intensities such as are achieved with cultured gametocytes in the standard membrane feeding assay (SMFA) [8-10], the progression from oocyst formation to mosquito infectivity is assumed. In MFA on naturally infected individuals, oocyst intensities within the range 1 to 5 are commonly observed [11-13], and it is currently not fully understood how these low oocyst densities relate to later sporozoite development [8]. Experiments that simultaneously detected intact oocysts and salivary gland sporozoites or compared oocysts and sporozoites in separate groups of mosquitoes suggest that not all oocysts contribute effectively to mosquito infectivity by releasing sporozoites into the haemocoel [14-17]. The impact of oocyst arrest in low

intensity infections might decrease the reliability of oocyst prevalence as an indicator of infectivity because it could lead to the total failure of sporozoite release.

To validate the development of whole-mosquito approaches to infection detection, we report the results of experimental assessments of the dynamics of *P. falciparum* oocyst-sporozoite development. Using antibody staining techniques we directly determine the extent of oocyst rupture in low intensity mosquito infections and in the same mosquitoes, assess the efficiency of sporozoite production or invasion of the salivary glands. The suitability of mosquito infection detection by both circumsporozoite protein (CSP) ELISA and *P. falciparum* specific 18s PCR was determined in comparison with routine microscopy

Results

Achieving low oocyst intensity infections

In line with previous observations [8, 18] oocyst prevalence and intensity were highly related (Fig. 1). Gametocyte culture material was diluted with uninfected blood to achieve oocyst intensities that are in line with the 1-5 oocysts typically observed in natural *P. falciparum* infections. Infection prevalence (measured at day 7 PI) for the separate experiments (n=30-104) ranged between 33% and 86.5%, with mean oocyst intensities from 0.57 and 4.7. In total 1591 mosquitoes were used in the current study (405 *A. stephensi*, 1186 *A. gambiae*). Overall median oocyst density in infected mosquitoes was 2 oocysts (IQR 1-5, range 1-10).

Measuring oocyst rupture in low intensity infections

To determine the extent of oocyst rupture in mosquitoes with a low oocyst intensity, intact oocysts were detected among sub-groups of a single cage of *A. stephensi* at days 7 (n=20), 15 (n=20), 17 (n=20), 21 (n=20), 24 (n=20) and 27 (n=20) PI**.** The prevalence of intact oocysts on days 7, 15, 17, 21, 24 and 27 was 100% (95% CI 96.4-100%), 45% (95% CI 23.1-68.5%), 25% (95% CI 8.7-49.1%), 25% (95% CI 8.7-49.1%), 40% (95% CI 19.1-63.9%) and 20% (95% CI 5.7-43.7%) respectively (see Fig. S1 in the Supplementary information online). Four experiments were subsequently conducted using antibody staining to identify ruptured as well as intact oocysts (Fig. 2). In the largest two experiments, oocyst prevalence and median intensity were first determined in groups of 30 mosquitoes by standard mercurochrome staining at day 7 PI, before a total of a total of 175 mosquitoes were dissected for antibody staining at days 14 and 21 PI (Fig. 3). For experiment 1, oocyst prevalence at day 7 PI was 43%, and median oocyst intensity in infected mosquitoes was 1 (IQR 1-2, range 1-5). In experiment 2, oocyst prevalence at day 7 was 80%, while median oocyst intensity for infected mosquitoes was 3 (IQR 1-4.3, range 1-10). No significant differences were observed in ruptured oocyst prevalence (the proportion of mosquitoes with ≥ 1 ruptured oocyst) or mean intensity between mosquito groups dissected at day 14

or 21 PI, indicating that oocyst rupture after day 14 PI was limited. Rupture and oocyst prevalence at all time points are given in Fig. 3. In the two smaller experiments (3&4) oocyst prevalence and median intensity were first determined in groups of 30 mosquitoes by standard mercurochrome staining at day 7 PI and oocyst rupture was subsequently determined between 11-16 days PI, reserving the mosquito carcass for assessments of sporozoite intensity. For experiment 3 (n=98), median oocyst intensity in infected mosquitoes was 2 (IQR 1-4, range 1-10), oocyst prevalence at day 7 was 80%, and the proportion of oocyst positive mosquitoes with at least one ruptured oocyst was 84.6% (66/78, 95% CI 74.7-91.8%). For experiment 4 (n=39), median oocyst intensity in infected mosquitoes was 2 (IQR 1-3, range 1-9), oocyst prevalence at day 7 was 77%, and the proportion of oocyst positive mosquitoes with at least one ruptured oocyst was 96.7% (29/30, 95% CI 82.8-99.9%). The prevalence of oocyst rupture within oocyst positive mosquitoes for experiments 1-4 is shown as Supplementary Fig. S2 online.

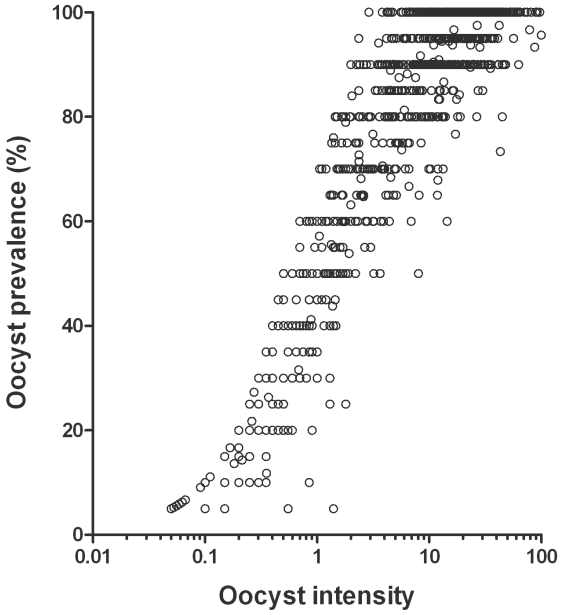


Figure 1. Oocyst prevalence and intensity for experimental feeds utilising the NF54 *P. falciparum* strain conducted in the mosquito infectivity unit at RUMC, Nijmegen between 2011 and 2013. Each data point represents the mean oocyst intensity and oocyst prevalence in a single group of mosquitoes. Criteria for inclusion in these figures were sample size (>10 mosquitoes), parasite strain (NF54 strain only) and mosquito species. Data were collected from both experimental and control feeds between 6 and 9 days PI. In total 1323 separate experimental feeds comprising 21240 mosquito dissections are shown (1233 experimental feeds with *A. stephensi*, 90 experimental feeds with *A. gambiae*).

Taken together, our results show that in mosquitoes where the majority of infections stemmed from 1-3 oocysts, 72% (390/542) of all oocysts had undergone rupture at the time of observation, while 66.7-96.7% of infected mosquitoes had at least one ruptured oocyst.

To examine the efficiency of sporozoite production and invasion of the salivary glands, groups of mosquitoes were examined for the detection of ruptured oocysts and quantification of sporozoites within the mosquito body (excluding the gut) (Fig. 4a) and the salivary glands (Fig. 4b). In mosquitoes in which sporozoites were detected in the body, a positive correlation between ruptured oocyst number and sporozoite intensity was observed (R^2=0.61, p<0.0001). Furthermore, all mosquitoes with detectable oocyst rupture were sporozoite positive, indicating that rupture will always result in a release of sporozoites into the mosquito haemocoel. In a single mosquito sporozoites were detected while no ruptured oocysts were detected by microscopy. It is probable in this instance that a ruptured oocyst was mistakenly identified as being intact. Considering all mosquitoes with ruptured oocysts, sporozoite intensity in the mosquito body ranged from 100-44600. Median production was 2000 sporozoites/ruptured oocyst (inter-quartile range (IQR) 950-3500). When considering only those cases where a single oocyst was found to have ruptured, the median number of sporozoites observed per oocyst was 2100 (IQR 1000-3750).

Sporozoite production and invasion of the salivary glands are uniformly effective in mosquitoes with low oocyst intensities

To investigate the efficiency of sporozoite production from ruptured oocysts, the (qualitative) presence of sporozoites released into the haemocoel was determined both by microscopy after antibody staining and by PCR in mosquitoes which had undergone midgut dissection for oocyst detection. By removing the mosquito midgut for oocyst detection, sporozoite detection was restricted to those released from the midgut into the mosquito body. Sporozoites were detected in all rupture positive mosquitoes by both microscopy and PCR (85/85). No sporozoites were detected in mosquitoes without evidence of rupture (0/17). Among the 66 mosquitoes which underwent salivary gland dissection, 45 out of 46 rupture positive mosquitoes were also gland positive (97.8%, 95% CI = 88.6-99.9%) (Fig. 4b). In another mosquito, 320 sporozoites were found while no ruptured oocysts were detected. The relationship between the intensity of ruptured oocysts and sporozoite intensity in the salivary glands was positive, but as for sporozoites in the mosquito body, not clearly linear (R^2=0.34, p<0.0001). Salivary gland sporozoite intensity ranged from 78-26600. The median number of sporozoites in the glands was 1250 per oocyst (IQR 313-2400). For single ruptured oocysts only median sporozoite intensity per oocyst was 1000 (IQR 256.5-2450).

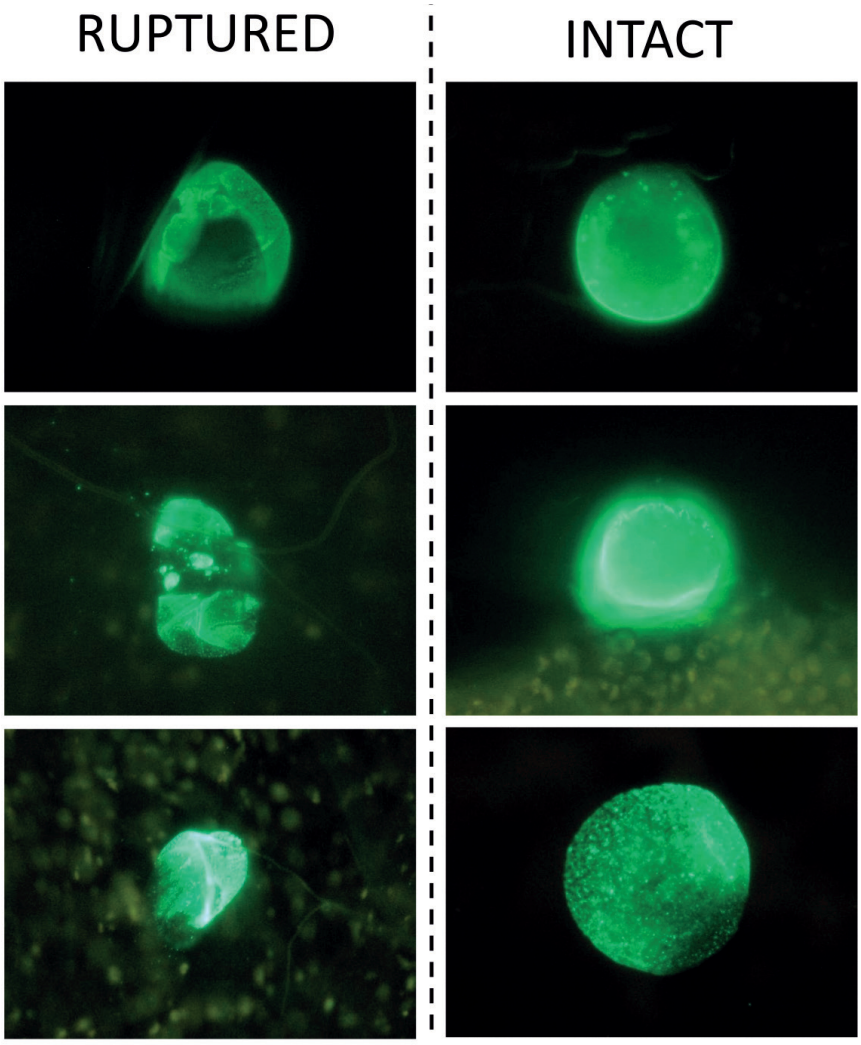
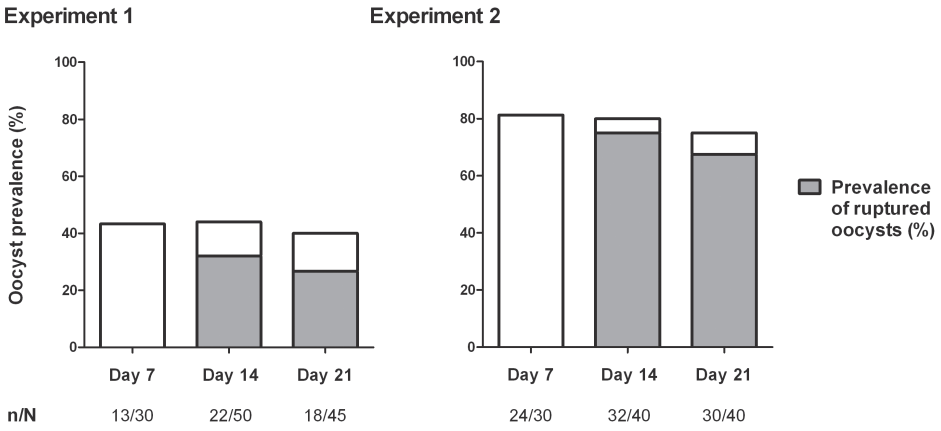


Figure 2. Classification of oocyst condition. Mosquito midguts were dissected in phosphate buffered saline (PBS) and stained using 3SP2-Alexa488 anti-CSP antibodies. After staining, midguts were washed twice with PBS for 10 minutes before being sealed under a glass cover slip with Vaseline petroleum jelly. All oocysts were identified and assigned a condition by two independent microscopists. Intact oocysts were visibly unbroken. Ruptured oocysts and oocyst remnants were visibly broken or degraded. All samples were examined within 8 hours of dissection, but sample condition remained stable for a number of days (stored at 4°C) prior to dissection for re-examination. Three ruptured and three intact oocysts are shown in the figure for demonstrative purposes.



Oocysts	Frequency
0	72
1	39
2	10
3	3
4	0
5	1
Total	125

Oocysts	Frequency
0	24
1	22
2	21
3	19
4	12
5	6
6	2
7	1
8	2
10	1
Total	110

Figure 3. Total oocyst prevalence and prevalence of oocyst rupture. n/N = oocyst positive mosquitoes/total number of mosquitoes dissected. Prevalence of oocyst rupture is given as the proportion of mosquitoes in which any oocysts were observed to have undergone rupture. Tables indicate the range and frequency of oocyst intensities observed in experiment 1 and 2. In experiment 1, 16/22 (72.7%, 95% CI = 49.8-89.3%) infected mosquitoes had at least one ruptured oocyst at day 14 PI, while 12/18 (66.7%, 95% CI= 41-86.7%) were rupture positive at day 21 PI. In experiment 2, 30/32 (93.8%, 95% CI 79.2-99.2%) infected mosquitoes were rupture positive at day 14 PI, while 27/30 (90%, 95% CI = 73.5-97.9%) were rupture positive at day 21 PI.

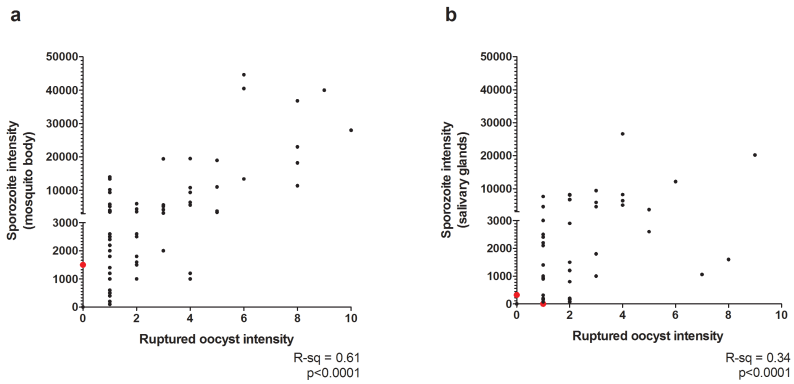


Figure 4. The number of sporozoites in the mosquito body or salivary glands, and ruptured oocyst intensity in the same mosquitoes. The detection limit for sporozoites detected in a single mosquito sample was 78 sporozoites (1 sporozoite observed in 64 Bürker chamber fields / 0.256µl homogenate). The Y-axis is split from 1-3000 sporozoites and from 3000-50,000 sporozoites because of the heterogeneous distribution of sporozoites within mosquitoes. Data points in red indicate erroneous observations (sporozoite +, rupture – or *vice versa*). **A.** Y-axis: Sporozoite total calculated from duplicate samples of whole homogenised mosquito (excluding gut) stained with 3SP2-Alexa488 conjugate antibodies. X-axis: Ruptured oocyst intensity. Median oocyst intensity in infected mosquitoes was 2 (IQR 1-4, range 1-10). The data point in red is a single mosquito for which 1500 sporozoites were found associated with no ruptured oocysts. **B.** Y-axis: Sporozoite total calculated from duplicate samples of homogenised 3SP2-Alexa488 stained salivary glands only. X-axis: Ruptured oocyst intensity. Median oocyst intensity in infected mosquitoes was 2 (IQR 1-3, range 1-9). The data points in red are a mosquito in which 320 sporozoites were found associated with no ruptured oocysts, and a mosquito in which a single ruptured oocyst was found associated with no sporozoites.

The application of oocyst prevalence as a high throughput read-out system

Having demonstrated that at the lowest levels of oocyst intensity oocyst rupture and sporozoite release occurs in the majority of mosquitoes, we sought to determine the utility of high throughput techniques for the detection of any mosquito infection at time points after which oocyst development may have occurred. Nine experimental feeds were conducted. At day 7 PI, 30 mosquitoes were dissected as standard for oocyst detection. Separate groups of mosquitoes were then taken from the same cages at days 7, 10 and 14 PI for individual homogenisation. Mosquito homogenate was processed by both CSP ELISA and 18s PCR (Table 1). Full details of these experiments are given in Supplementary Table S1 online. In total 796 mosquitoes were tested by both CSP ELISA and 18s PCR. When compared to microscopy, ELISA underestimated mosquito infection rates in 100.0% (8/8) of experiments when ELISA was performed on day 7 and in 11.1% (1/9) of experiments when ELISA was performed on day 10. When ELISA was performed on day 14, there was no underestimation of infection rates compared to microscopy and 22.2% (2/9) of

experiments had higher mosquito infection estimates by ELISA compared to microscopy (Table 1). By contrast, mosquito infection prevalence at day 7 determined by 18s PCR and microscopy did not significantly differ in any of the 8 experiments (experiment 8, p=0.054), while at day 10 and 14 PI PCR prevalence was significantly higher than microscopy prevalence in 1 and 2 experiments respectively. In the two day 14 experiments where prevalence was higher by both ELISA and 18s PCR than by microscopy, differences in paired ELISA and PCR measures were non-significant or borderline significant (Day 14 Experiment 7, p=0.046; Day 14, Experiment 8 p=0.317).

Relative to positivity by PCR, the sensitivity of CSP ELISA for the detection of *P. falciparum* in whole homogenised mosquitoes increased from 6.8% at day 7 PI to 61.7% at day 10, and 86.7% at day 14 PI. 28/796 mosquitoes (3.5%) were determined as positive for CSP but negative for *P. falciparum* by PCR. ELISA OD values for these mosquitoes varied widely above the cut off for positivity of OD 0.12 (median OD (IQR) = 0.336 (0.14-0.91). Of the 256 mosquitoes determined negative by ELISA but positive by PCR (32.2% of total), 64.1% (164/256) were mosquitoes collected on day 7 PI, and 28.5% (73/256) were collected at day 10 PI. Agreement values between ELISA and PCR were 40.0% at day 7 PI (Kappa 0.02, p=0.15), 71.2% at day 10 PI (Kappa 0.45, p>0.0001) and 85.3% at day 14 PI (Kappa 0.69, p>0.0001). Between paired measures positivity rates by PCR and ELISA were significantly different at day 7 (p<0.0001) and 10 PI (p<0.0001) but not at day 14 PI (p=0.49).

Table 1. The results of nine experimental feeds where oocyst prevalence was determined in a sample of mosquitoes at day 7 PI, and infection prevalence was subsequently determined in separate samples collected 7, 10 and 14 days PI by both CSP ELISA and 18s PCR

Exp. ID	Microscopy Prevalence % (n/N)	CSP ELISA Prevalence % (n/N)			18s PCR Prevalence % (n/N)		
	Day 7 P1	*Day 7 PI*	*Day 10 PI*	*Day 14 PI*	*Day 7 PI*	*Day 10 PI*	*Day 14 PI*
1	63.3 (19/30)	2.5* (1/40)	17.5* (7/40)	53.8 (21/39)	66.7 (26/39)	60 (24/40)	64.1 (25/39)
2	36.7 (11/30)	0.0* (0/40)	36.6 (15/41)	37.5 (15/40)	37.5 (15/40)	46.3 (19/41)	32.5 (13/40)
3	83.3 (25/30)	13.3* (4/30)	70 (21/30)	70 (7/10)	73.3 (22/30)	83.3 (25/30)	90 (9/10)
4	86.7 (26/30)	20.7* (6/29)	59.1 (13/22)	85.7 (12/14)	69 (20/29)	90.9 (20/22)	78.6 (11/14)
5	53.3 (16/30)	0.0* (0/30)	39.3 (11/28)	54.5 (12/22)	66.7 (20/30)	50 (14/28)	40.9 (9/22)
6	70 (21/30)	10.0* (3/30)	43.3 (13/30)	50 (12/24)	70 (21/30)	70 (21/30)	58.3 (14/24)
7	66.7 (20/30)	4.9* (2/41)	71 (22/31)	79.2 (19/24)	68.3 (28/41)	96.8* (30/31)	95.5* (21/22)
8	33.3 (10/30)	0.0* (0/41)	36.4 (12/33)	63.6* (28/44)	58.5 (24/41)	54.5 (18/33)	68.2* (30/44)
9	50 (15/30)	nd	43.3 (13/30)	87.5* (14/16)	nd	70 (21/30)	62.5 (10/16)

n/N Number of mosquitoes positive (Microscopy/ELISA/PCR) / total sample size
PI Post-infection
* Significant difference in prevalence compared with oocyst prevalence at day 7 PI (P-value <0.05, Chi-squared test)
nd Not done

Colours are demonstrative of relative differences in prevalence between all time-points and methods (dark red = High prevalence, light red = low prevalence).

Discussion

The proportion of mosquitoes that become infective is the most relevant outcome measure for assessments of the human infectious reservoir for malaria and for evaluating the impact of transmission reducing interventions on human infectivity. If the extent of oocyst non-rupture in mosquitoes with low oocyst intensities is not prohibitively high, and if sporozoite release and invasion of the salivary glands is effective, then the detection of oocysts, regardless of their density, provides an acceptable estimate of mosquito infectivity for high throughput assessment. In this study we show that the majority of low density infections result in oocyst rupture, sporozoite release and colonisation of the mosquito salivary glands. We further show that PCR but not CSP ELISA may replace microscopy without a loss in sensitivity when examining mosquitoes on day 7 post infection.

The first aim of the current study was to establish the usefulness of oocyst prevalence as a stand-alone indicator of infectivity for the evaluation of MFA. The transition from oocyst formation to sporozoite transmission to humans was recently highlighted by Churcher *et al.* as a major gap in our current understanding and limitation to the to the evaluation of TRI [8]. The valuable few studies which have investigated the dynamics of late sporogony are summarised by Vaughn *et al.* [17]. A common observation among these studies is the presence of intact oocysts when most others have ruptured [14-16]. Rates of oocyst arrest have only been recorded in detail for *P. vivax* by Zollner *et al.,* who estimated that sporozoite production reached only 11% of its theoretical potential by day 18 PI, due to sub-optimal productivity by some oocysts or their total failure to release sporozoites [15]. Though non-rupture of a proportion of oocysts is not necessarily relevant where oocyst intensity is high, its occurrence in low intensity infections may significantly affect the accuracy of infectivity estimates based on oocyst prevalence alone. By visualising ruptured oocysts, we provide direct evidence that in low density infections (median oocysts in infected mosquitoes = 2, IQR 1-5) rupture occurs in the majority of *P. falciparum* infections. Combining the results of 4 independent experiments, 85.7% (180/210) of mosquitoes with low density infections had at least one ruptured oocyst when measured between 11-21 days post infection. Our data support previous estimates of the efficiency of sporozoite production and salivary gland invasion by directly relating these measures to the intensity of oocyst rupture measured in the same mosquitoes, showing that infectivity after sporozoite release is almost uniformly effective. Following oocyst rupture, sporozoite production appears to be highly effective; 204 of the 205 mosquito's containing ruptured oocysts were also sporozoite positive outside the midgut. In the current study, gland invasion was achieved in 45/46 mosquitoes with evidence of oocyst rupture. The one instance where rupture was detected and salivary gland sporozoites were not may have arisen because sporozoites from this oocyst may not have successfully reached the salivary glands [14, 16], or because rupture occurred so close to the time of dissection that haemocoel traversal was ongoing [19, 20].

Previous estimates of the productivity of *Plasmodium* oocysts have been restricted either because estimates were carried out artificially (excising oocysts from midguts and counting the sporozoites within) [21, 22], or because they were based on the observation of intact oocysts without consideration of the proportion which might fail to rupture [14-16, 23]. In the current study, our direct approach to oocyst quantification and classification allowed for more precise estimates of *per capita* potential and effective sporozoite production. For each ruptured oocyst a mean of 2000 sporozoites were found in the mosquito body while 1250 were observed in the salivary glands, suggesting that the efficiency of gland invasion is ~60% of the total number of sporozoites released. Had oocysts been considered irrespective of their condition, per oocyst sporozoite numbers would have been reduced by approximately 28%, to 1440 in the body and 900 in the salivary glands (72% of all oocysts that were observed on mosquito midguts ruptured). Encouragingly, these estimates very closely match previous observations in the same species combination (1361 salivary gland sporozoites per oocyst, based on data from 33 mosquitoes) [23]. The results of the current study indicate that though the overall efficiency of sporozoite production, release and traversal may be relatively low, the effectiveness is nonetheless extremely high with the vast majority of oocyst-positive mosquitoes eventually becoming sporozoites positive. There is no obvious bottleneck preventing the establishment of salivary gland invasion where they are released.

As oocyst intensities in the current study were manipulated to exist over a very narrow range (>80% 1-3 oocysts, range 1-10), analysis of the relationship between rupture rates and oocyst intensity was not possible. Though it is likely that at high oocyst intensities competition for nutrients may inhibit rates of oocyst maturation and rupture [15, 24, 25], we consider it unlikely over the range of oocyst intensities observed in the current study that rupture rates would have decreased significantly with oocyst intensity, especially since a second bloodmeal was given to increase availability of nutrients and better mimic natural mosquito feeding frequencies.

For evaluations where mosquito infections levels are naturally low, such as for studies that aim to determine the human infectious reservoir for malaria or determine the impact of transmission reducing interventions for which the mode of action is before oocyst formation (e.g. gametocytocidal drugs or pre-fertilization and anti-ookinete transmission-blocking vaccines), we consider oocyst prevalence a reliable, albeit not perfect, indicator of mosquito infectiveness. Since the majority of oocyst positive mosquitoes had at least one ruptured oocyst from which sporozoites reached the salivary gland, and as the number of sporozoites egested during probing (<100) is considerably less than the number produced by single oocysts (1,359-14,000) [21, 22, 26-28] and independent of total salivary gland sporozoite intensity [28], we consider it plausible that infectivity can be predicted with reasonable certainty from oocyst prevalence. The modest overestimation of infectivity that is a consequence of some oocysts not rupturing in low density infections would

be acceptable for the evaluation of programs aiming to interrupt parasite transmission. However, this overestimation may require consideration in studies where direct effects on sporozoite development or invasion of the salivary glands may be responsible for variations in the proportion of mosquitoes that are sporozoite-positive [29].

Using oocyst prevalence as the sole outcome measure for mosquito feeding assays has advantages in terms of mosquito husbandry, the number of mosquitoes surviving long enough to contribute to read out measures and laboratory safety. As such, the results of the current study open the way for the replacement of microscopy with high throughput techniques for oocyst detection to allow for the large number of mosquito observations needed to reliably estimate TRI efficacy [8]. We explored two scalable options: CSP-ELISA and 18s PCR. PCR and ELISA have previously been used for the detection of midgut oocysts [24, 30] or salivary gland sporozoites [31-34] in dissected colony and wild caught mosquitoes. In the current study, the same homogenate from undissected mosquitoes was used for both PCR and CSP-ELISA. CSP-ELISA underestimated mosquito infection rates compared to microscopy and PCR, especially at day 7 PI when sporozoite production and antigen release from oocysts may have been limited [19]. Several approaches were explored for mosquito homogenisation and maximization of CSP release from midgut oocysts including sonication, rapid freeze-thaw cycling, and preparation with various detergents. None of these approaches solved the apparent lack of CSP release from PCR-detectable oocysts (data not shown) that precludes the use of CSP-ELISA as read-out for mosquito feeding assays before day 10 PI. In contrast, 18s PCR showed good agreement with microscopical oocyst prevalence at all time points. Where statistical differences in prevalence were observed these were because of higher prevalence by PCR, suggesting that microscopy may have missed a small number of (low density) infections. Day 14 CSP-ELISA results supported PCR estimates in experiments where PCR infection prevalence was higher than microscopy prevalence, suggesting that differences in oocyst prevalence between samples may also have affected these outcomes. The earliest time point at which the whole-mosquito approach to oocyst detection can be applied depends upon the rate at which non-established parasites are cleared from the digestive tract of mosquitoes following the infective blood meal. Bell and Ranford-Cartwright estimated using PCR based on the amplification of the MSA-1 gene that *Plasmodium* DNA can no longer be detected in the mosquito body ≥5 days PI [24]. Using the current technique, this creates a boundary at 5 days PI after which any *Plasmodium* in mosquitoes represents an established infection. Ookinete specific markers could bring forward this boundary, but to establish the utility of such markers the efficiency of ookinete-oocyst transition would require further investigation; current estimates indicate that in *Plasmodium* species affecting humans population loss during the ookinete-oocyst transition may be between 50-75% [17].

The results of the current study validate the use of oocyst prevalence as an indicator of infectivity. Sensitive PCR based approaches to the detection of mosquito infection

at time points after oocyst formation are capable of replicating the results of standard microscopical oocyst prevalence assessments.

Methods

Mosquito infections

i) Mosquito rearing and parasite culture

Anopheles stephensi (Sind-Kasur Nijmegen strain) [35] and *A. gambiae* (Ngousso strain) [36] were reared at 30°C and 70-80% humidity, while exposed to a 12/12 hour day/night cycle. Direct comparisons of SMFA experiments using *A. stephensi* and *A. gambiae* observed no differences between mosquito species in the association between oocyst prevalence and oocyst density or estimates of transmission reduction of transmission-blocking monoclonal antibodies or field sera (Eldering *et al.*, in preparation). For the current experiments, *A. stephensi* mosquitoes were used for all experiments on sporogonic development. The experiments on high-throughput assessment of infected mosquitoes by PCR or ELISA used *A. gambiae* mosquitoes to allow a direct comparison with field-based mosquito feeding assays. Mature *P. falciparum* (NF54) gametocytes (14 day culture, 0.3-0.5% gametocytes, 2% haematocrit) were obtained from an automated tipper system and prepared as previously described [37, 38]. To achieve low intensity infections for examinations of oocyst condition and sporozoite production, infective blood meals that are routinely used and produce high infection prevalence (averaging >70% with oocyst intensities in infected mosquitoes >10) were diluted at a ratio of 1:10 with uninfected blood. Dilutions between 1:5 and 1:20 were used for comparisons of microscopy, ELISA and PCR for the detection of infection among groups of mosquitoes, where we aimed for a range of infection intensities (see Supplementary Table S1 online).

ii) Mosquito feeding and dissections

3-5 day old *A. stephensi* mosquitoes were fed on a glass membrane midi-feeder system containing ~1.25ml of the *P. falciparum* culture mix [37, 38]. Unfed and partially fed mosquitoes were removed after feeding and blood fed females were maintained at 26°C and 70-80% humidity. For examinations of oocyst condition and sporozoite production, mosquitoes received an additional uninfected human blood meal between 7 and 10 days PI [39]. Routine staining of midguts was done in 1% mercurochrome.

Immunostaining experiments

i) Oocyst prevalence, rupture and sporozoite prevalence at different time-points post-infection

To examine oocyst condition and sporozoite prevalence at different time-points PI, the midguts of 30 *A. stephensi* mosquitoes were removed at day 7 PI, stained with mercurochrome, and microscopically examined for oocysts prior to rupture. At both days 14 and 21 PI, two groups of mosquitoes were dissected (175 observations total) for oocyst and sporozoite examination by antibody staining, ensuring that in each of the four replicates approximately 20 positive mosquitoes were examined. At these later time-points midguts were dissected in 20µl phosphate buffered saline (PBS, pH 7.2), transferred to a fresh drop of PBS and stained using 3SP2-Alexa488 anti-CSP antibodies (1:400) for 30 minutes at room temperature (RT) in a humid container. Details of the 3SP2 antibodies used for oocyst/sporozoite staining and CSP ELISA (produced in Nijmegen, Netherlands) have been previously described [28, 40]. After staining, midguts were washed twice with PBS for 10 minutes before being sealed under a glass cover slip with Vaseline petroleum jelly (Unilever, UK). During all dissection and washing steps care was taken to avoid artificial oocyst rupture. Intact/degenerated and ruptured oocysts were counted using an incident light fluorescence microscope. Mosquito carcasses and the PBS from the first dissection step were homogenised in glass grinding tubes with 180µl of PBS. Two lots of 15µl of homogenate were transferred to a glass slide, incubated with 5µl of 3SP2-Alexa488 conjugate (1:400), and left for 30 minutes at RT in a humid container. After staining, cover slips were applied with Vaseline and sporozoite positivity was determined by examining 50 fields for each sample (100 fields per mosquito) using an incident light fluorescence microscope.

ii) The number of sporozoites released by ruptured oocysts

To estimate the contribution of ruptured oocysts to sporozoite intensity in the whole mosquito body, the midguts of 98 *A. stephensi* mosquitoes were dissected and stained between days 11-14 PI using 3SP2-Alexa488 antibodies as described above. Mosquitoes were collected across four feeding experiments, in which mosquito homogenate was transferred to counting chambers (Bürker, Labor Optik, UK) and allowed to settle for 20 minutes in a humid container before quantification was conducted using an incident light fluorescence microscope.

iii) The number of sporozoites invading the salivary glands following oocyst rupture

To determine the association between oocyst rupture and mosquito infectivity, the midguts and salivary glands of 66 *A. stephensi* mosquitoes were carefully dissected between days 12-16 PI. Mosquitoes were collected across two feeding experiments. Salivary glands were

removed from the mosquito carcass and homogenised in glass grinding tubes with 20µl of 3SP2-Alexa488 conjugate (1:400). Sporozoite quantification was then conducted as described for body sporozoites.

High throughput mosquito processing and infection detection

i) Mosquito processing and experimental design

Nine cages of approximately 160 *A. gambiae* mosquitoes were infected as described above for investigation of the comparative efficacy of microscopy, ELISA and PCR in detecting mosquito infection prevalence. Alongside standard dissections carried out on a sample of mosquitoes at day 7 PI, CSP-ELISA and PCR were performed on each mosquito in groups collected from each cage at day 7, 10 and 14 PI. Details of sample sizes (n=10-44) are in Table 1, and full details of all results are in Supplementary Table S1 online.

ii) CSP-ELISA

CSP ELISA was performed as previously described, with minor modifications [41]. Mosquitoes were homogenised in 250µl phosphate buffered saline (PBS pH 7.2) solution with 1% sarcosil. Sterilin ELISA plates were coated with 3SP2 (Nijmegen, Netherlands) at 5µg/ml, diluted in PBS. Homogenate of test mosquitoes were analysed in duplicate, alongside blank wells (just sample diluent) and a standard curve of recombinant CSP (Gennova, 0.1 µl/ml). A selection of homogenates from 30 uninfected blood-fed control mosquitoes were tested on every plate, so that each sample was tested at least 5 times over the course of the experiment. A cut off for CSP positivity was determined at an optical density (OD) of 0.12, which was the mean OD of the uninfected mosquitoes plus three standard deviations.

iii) DNA extraction and 18s PCR

Phenol-chloroform DNA extractions were carried out on the 150µl of mosquito homogenate remaining from CSP ELISA as described previously, with minor modifications (23). Briefly, Proteinase K was added to each sample of mosquito homogenate at 20mg/ml, and samples were incubated overnight at 55°C. After incubation, samples were briefly spun down and a Phenol/Chloroform/Isoamyl alcohol (25:24:1) mix was added to each sample at a ratio of 1:1. Samples were mixed vigorously and centrifuged at 10,000g for five minutes. After spinning, the aqueous layer was mixed with 250µl of Isopropanol, and 100 µl of a mix of 3M Sodium Acetate (pH 5.2), Glycogen (10mg/ml), and nuclease free water. Samples were then mixed repeatedly during a 15 minute incubation at RT, before centrifugation at 13,000g for 30 minutes at 4°C. Supernatant was removed, washed with 1ml of 70% Ethanol, and spun again for 5 minutes at 4°C. Ethanol was then removed, and the pellet dried and suspended in 20µl of nuclease free water.

PCR targeting the small sub-unit ribosomal RNA (ssrRNA) gene of *P. falciparum* was performed as previously described, with minor modifications (24). The volume of DNA used in the nest 1 reaction was increased from 1µl to 5µl, and the volume of nest 1 template used in the nest 2 PCR was adjusted to 2µl. For a more detailed overview of primer sequences, controls and PCR cycling conditions see Baidjoe et al. 2013[42]. N1 and N2 products were mixed and 10µl was visualized on 1.5% agarose gel by electrophoresis in 0.5X Tris-acetate-EDTA buffer (0.04 M Tris-acetate and 1 mM EDTA, pH 8.0).

Data analysis

Statistical analysis was conducted using STATA 12 (StataCorp., TX, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). In the analyses of oocyst prevalence and condition, and between groups of mosquitoes analysed by dissection and PCR or ELISA, differences in prevalence between groups were tested by Chi-squared test. Differences between paired PCR and ELISA positivity values were tested by McNemars' Chi Square test. For agreement between PCR and ELISA carried out on the same mosquitoes, sensitivity, specificity, agreement and kappa values were presented using PCR for reference. Associations between ruptured oocyst intensity and sporozoite intensity were quantified with Spearmans correlation coefficients. *Per capita* sporozoite production (for both whole mosquito and salivary gland specific estimates) was presented as the mean of the sporozoite production per mosquito divided by the number of ruptured oocysts observed within the same mosquito.

Acknowledgments

We would like to thank Jacqueline Kuhnen, Laura Pelser-Posthumus, Astrid Pouwelsen, and Jolanda Klaassen for conducting all mosquito breeding, and for their assistance in the evaluation of mosquito membrane feeding experiments. The work of WS, KL, CJD and TB is supported by grants from the Bill & Melinda Gates Foundation (AFIRM, grant number OPP1034789) and PATH Malaria Vaccine Initiative (MVI). TB is further supported by European FP7 project REDMAL (#242079) and a Marie Curie Career Integration Grant from the European Community's Seventh Framework Programme (SIGNAL, PCIG12-GA-2012-333936). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Selected supplementary material

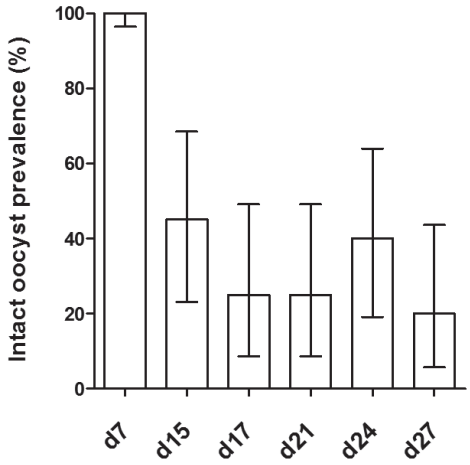


Figure S1. The prevalence of midgut oocysts in mosquito groups from the same experimental feed examined by standard dissection between days 7 and 27 PI. Oocyst prevalence was determined by microscopical examination of mercurochrome stained midguts at all time points (n=20 per time point, n=120 in total). Typically 10-15 mosquitoes per group are dissected as an immediate measure of oocyst prevalence and mean intensity during the SMFA. Oocysts identified here are likely to have been intact, though the possibility that some may have undergone various degrees of rupture or degradation cannot be excluded. Error bars represent 95% confidence intervals (CI). On day 7, median oocyst intensity was 2 (IQR 1-2, range 1-6).

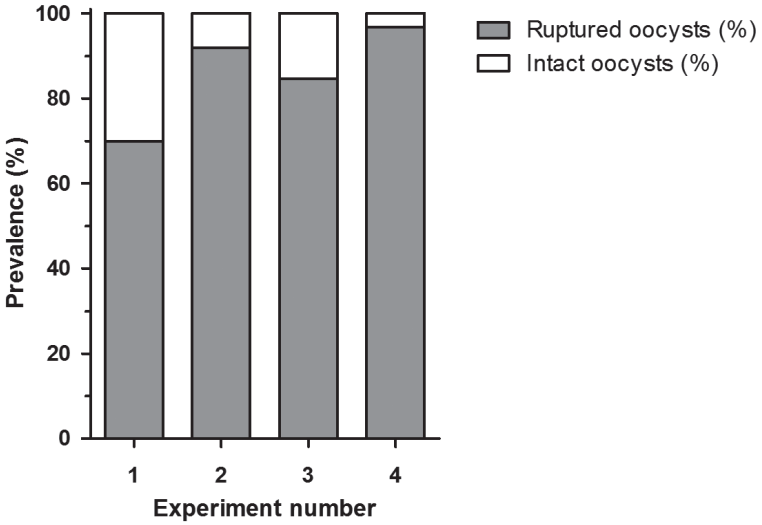


Figure S2. The prevalence of oocyst rupture in infected mosquitoes. Prevalence of oocyst rupture is given as the proportion of mosquitoes in which any oocysts were observed to have undergone rupture. Prevalence of intact oocysts is given as the proportion of mosquitoes in which only intact or degenerated oocysts were observed. All oocyst examination time-points have been combined within experiments, so that experiments 1 and 2 represent the prevalence of oocyst rupture in mosquitoes examined on day 14 and 21 PI, and experiment 3 and 4 the prevalence of oocyst rupture in mosquitoes examined between 11 and 16 days PI. Details of sample numbers are in the text, and in the legend for Fig. 3. Total prevalence of oocyst rupture was 70% (95% CI 53.5-83.4%) in experiment 1, and 91.9% (95% CI 82.2-97.3%) in experiment 2.

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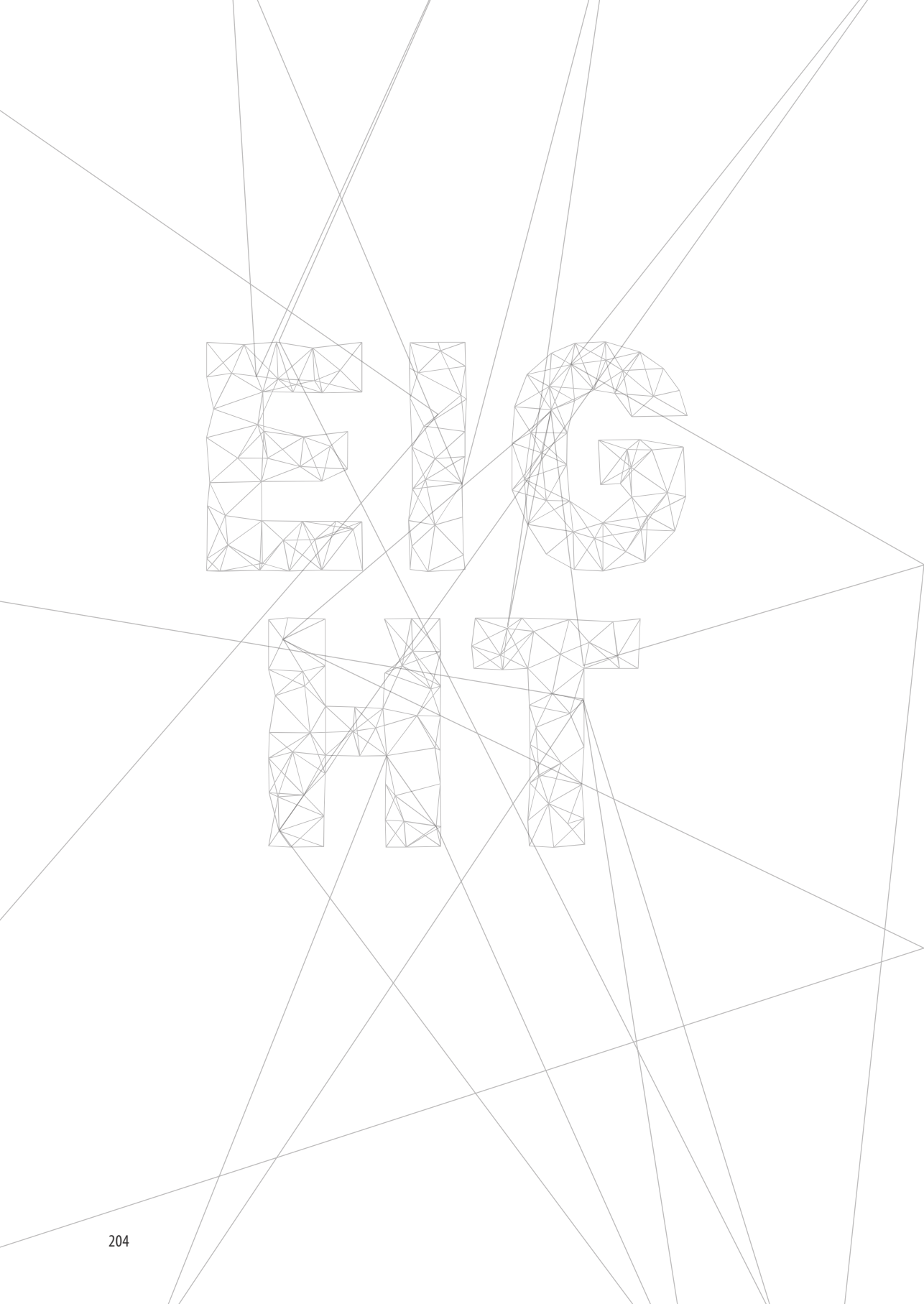

Chapter 8

A comparison of *Plasmodium falciparum* circumsporozoite protein-based slot blot and ELISA immuno-assays for oocyst detection in mosquito homogenates

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Malaria Journal. 2015;14(1):451

Abstract

Background

The infectivity of *Plasmodium* gametocytes is typically determined by microscopically examining the midguts of mosquitoes that have taken a blood meal containing potentially infectious parasites. Such assessments are required for the development and evaluation of transmission-reducing interventions (TRI) but are limited by subjectivity, technical complexity and throughput. The detection of circumsporozoite protein (CSP) by enzyme-linked immunosorbent assay (ELISA) and enhanced chemiluminescent slot-blot (ECL-SB) may be used as objective, scalable alternatives to microscopy for the determination of infection prevalence.

Methods

To compare the performance of the CSP ELISA and ECL-SB for the detection of mosquito infection, four groups of *Anopheles stephensi* mosquitoes were infected with cultured *Plasmodium falciparum* gametocytes. At day-8, post-infection (PI) parasite status was determined by microscopy for a sample of mosquitoes from each group. At days 8 and 10 PI, the parasite status of separate mosquito samples was analysed by both CSP ELISA and ECL-SB.

Results

When mosquito samples were analysed eight days PI, the ECL-SB determined similar infection prevalence to microscopy; CSP ELISA lacked the sensitivity to detect CSP in all infected mosquitoes at this early time point. When mosquitoes were analysed 48 hours later (ten days PI) both assays performed as well as microscopy for infection detection.

Conclusions

Whilst microscopical examination of mosquito guts is of great value when quantification of parasite burden is required, ECL-SB and CSP ELISA are suitable alternatives at day 10 PI when infection prevalence is the desired endpoint, although CSP ELISA is not suitable at day 8 PI. These results are important to groups considering large-scale implementation of TRI.

Background

Transmission-reducing interventions (TRIs) that specifically aim to interrupt the transmission of malaria from man to mosquito form important components of malaria control and elimination strategies [1, 2]. The development, targeting and evaluation of TRIs requires robust assessments of the infectivity of human malaria infections to mosquitoes [3, 4]. Mosquito infection status is typically determined by microscopy following the observation of *Plasmodium* oocysts in the midgut or sporozoites in the salivary glands [5, 6]. Oocysts develop on the basal lamina of the mosquito midgut approximately two days after the ingestion of a blood meal containing infectious gametocytes, and can be visually detected by microscopy approximately six days post infection (PI) [5]. Sporozoites develop from the budding of sporoblasts in the developing oocyst, which give rise to hundreds or thousands of sporozoites in an explosive population expansion that starts seven to eight days PI [7, 8]. Beyond ten days PI, sporozoites rupture the oocyst capsule and enter the haemolymph, beginning their migration to the mosquito salivary glands [7, 9]. Within 8 hours of their release, sporozoites must invade the salivary glands or else be broken down in the mosquito haemolymph [10]. Sporozoites that succeed in entering the salivary glands are detectable from about 11 days PI, and the number in the glands appears to plateau after approximately 14 days PI [11] where they remain viable for long periods [12, 13]. Because of this stability and because very few sporozoites are egested during blood feeding [14, 15] it is generally accepted that a mosquito with any number of salivary gland sporozoites is infective to humans.

The goal of TRIs is to reduce the proportion of mosquitoes becoming infectious after taking a blood meal on vaccinated or treated individuals [16]. A recent study showed that eventual infectivity can be predicted with reasonable certainty from the detection of maturing oocysts in low-intensity infections [11]. With highly trained technicians, mosquito dissections can be performed quickly; however, the ability to properly identify and quantify developing oocysts is a specialized skill that requires long periods of training. Furthermore, the necessity to screen individual midguts during a limited time-window PI limits the throughput of mosquito feeding assays. It is highly desirable that the endpoint for efficacy assessments of TRIs be unambiguous, flexible with regard to the timing of mosquito processing, and usable by non-specialized staff. Screening mosquitoes for oocyst stage infections by high throughput immunological or molecular tools may provide an alternative to microscopy for processing large volumes of mosquitoes.

Circumsporozoite protein (CSP) is a ~60kD glycosylphosphatidyl-inositol (GPI)-anchored sporozoite, surface-coat protein with roles in parasite development in oocysts [17], traversal of the haemocoel [18], recognition and binding to the salivary glands [19, 20], protection after egestion into the human microvasculature [18], and invasion of human hepatocytes [21, 22]. In the mosquito, CSP is abundantly expressed by the developing parasite

[23], making the protein an ideal target for immuno-assays. The colorimetric enzyme-linked immunosorbent assay (ELISA) is commonly used for the detection of *Plasmodium* CSP in wild-caught mosquitoes [6, 24–26], where salivary gland infection (measured by homogenization of the head or head and thorax) has generally been the endpoint of interest. A positive ELISA test result indicates that mosquitoes ingested infectious parasites more than 11 days prior to assessment. If the ELISA is performed at seven days PI, when oocysts are visible by microscopy, CSP expression by developing oocysts is too low for detection [11]. The enhanced chemi-luminescent ELISA (ECL ELISA) and slot-blot assays (ECL-SB) were designed to overcome these issues of sensitivity, and are capable of detecting as little as 4.4 pg and 1 pg of recombinant CSP, respectively [27, 28], which could allow for *Plasmodium* detection shortly after the onset of CSP production during oocyst development.

Here, two immunoassays, the CSP ELISA and ECL-SB, were evaluated for their ability to detect CSP in mosquito homogenates processed from whole mosquito carcasses, by comparison to mosquito samples analysed by standard dissection and oocyst enumeration. Gametocyte cultures were diluted to ensure parasite burden in test mosquitoes was close to the level of *Plasmodium*-infected vectors from endemic regions [29, 30], providing groups of mosquitoes with controlled, but varying infection prevalence to investigate the performance of the two assays relative to microscopy for the detection of oocyst-stage infections. Since CSP expression increases during oocyst maturation [27, 31], the sensitivity of the assays in longer term infected mosquito specimens that contain a higher amount of CSP was also examined.

Methods

Infection of Anopheles stephensi mosquitoes with Plasmodium falciparum parasites

i) Mosquito rearing: Anopheles stephensi (Sind-Kasur Nijmegen strain) [32] were reared at 30°C and 70-80% humidity, while exposed to a 12/12 hour day/night cycle.

ii) Parasite culture: Mature *Plasmodium falciparum* (NF54) gametocytes (14-day culture, 0.3-0.5% gametocytes, 2% haematocrit) were obtained from an automated tipper system and prepared as previously described [33, 34]. To achieve low-intensity infections, infective blood meals that are routinely used and produce high infection prevalence (averaging >70% with mean oocyst intensities in infected mosquitoes >10) were diluted at a ratio of 1:10 with uninfected blood.

iii) Mosquito feeding assays: Four separate batches of gametocyte material were each fed to multiple cages of mosquitoes (10 cages total). For each cage, approximately 150 three- to five-days old *Anopheles stephensi* mosquitoes were fed on a glass membrane midi-feeder system containing ~1.25 ml of the *P. falciparum* culture mix [33, 34]. Unfed and partially

fed mosquitoes were removed after feeding and blood-fed females were maintained at 26°C and 70-80% humidity.

iv) Experimental design: After infection, mosquitoes were combined to have four large batches of mosquitoes that allowed examination by microscopy, CSP ELISA and ECL-SB. At day 8 PI, 20-30 mosquitoes per batch were examined for oocysts by microscopy. At days 8 and 10 PI, 36-48 mosquitoes per batch were stored at -20°C in sealed containers until analysis by CSP ELISA and ECL-SB. Sample sizes for the two assays were maximised based on the availability of live mosquitoes at the two time points and were kept uniform between the two assays. Leftover mosquitoes were killed and discarded. Full details of mosquito sample sizes for all assays and groups are in Table 1.

Microscopy

Routine staining of midguts for oocyst detection was done in 1% mercurochrome, as described previously [11, 33]. All oocyst detection was performed once by expert staff at Radboud UMC, Nijmegen, The Netherlands.

ECL slot-blot

i) Preparation of whole mosquito homogenates: CSP expression on the developing oocysts was determined in lysed whole mosquitoes using a procedure described previously [27]. Briefly, individual blood-fed or unfed mosquitoes were placed into single tubes and homogenized with a piston in 50 µl of lysis buffer (1X TBS, 0.5% SDS). The lysates were subsequently vortexed for 20 sec and then boiled for 5 min. The insoluble material was pelleted via centrifugation and the supernatant was collected and analysed.

ii) mAb 2A10: Anti-Pf CSP mAb 2A10 was generated using a hybridoma cell line acquired from the MR4/ATCC, Virginia, USA. A commercial source was used to produce ascites in mice and purify antibodies by Protein G affinity chromatography (Harlan Laboratories Inc. Madison, WI, USA). mAb 2A10 (1.55 mg/ml) immune-reactivity was characterized in IFA using *P. falciparum* sporozoites and in ELISA and Western Blot using rPf CSP.

iii) Performance of the ECL-SB: This assay was performed using a Minifold 48 slots, Whatman apparatus (GE Healthcare Life Sciences, 10447941; Piscataway, NJ, USA) in a slightly modified version of the standard protocol [27]. The ECL-reagents used in this assay were purchased as a kit (Life Technologies, Western-StarTM Immunodetection System, T1046, Grand Island, NY, USA). Approximately 20 µL of each sample lysate was loaded into each slot-blot well. Sample proteins were allowed to adsorb onto the nitrocellulose membrane for one hour and then slots were washed with 500 µl of deionized water four times. The membrane was next blocked at room temperature (RT) in iBlock blocking buffer (Applied Biosystems, T2015, Foster City, CA, USA) for one hour before being probed with anti-Pf CSP mAb 2A10 (at 0.31 µg/ml) for an additional hour. The membrane was subsequently washed three times (5 min each) in deionized water and three times in iBlock

blocking buffer. After the washings, the membrane was incubated with an AP-conjugated ECL-goat anti-mouse- IgM+IgG secondary antibody (1:5,000 dilution) for one hour at RT and then washed again as described following incubation with mAb 2A10. Before development, the membrane was rinsed twice for two minutes with 25 ml of 1X assay buffer and bands were visualized by incubating the membrane in 6 ml of ECL-substrate solution at RT for 5 min and exposure to an AR film (Kodak X-OMAT 1000A).

iv) ECL slot blot data acquisition and analysis: The band profile on the developed film was scanned and analysed using the ImageJ program [35]. The integrated optical density (IOD) of each band was determined by measuring the band intensity in a 'gated area'. The dimensions of the gated area for IOD determination was kept constant for each band on the scanned image. The capacity of a single blot was 48 mosquito samples, including three to five negative controls for the calculation of CSP positivity. Each blot was developed separately. As there were large variations in IOD values between blots (Figure 1), positivity determination for test mosquitoes was blot-specific, based on the mean IOD values of the negative controls processed in each blot. As the total number of mosquitoes processed at each time point in groups 2 and 4 exceeded the capacity of a single blot (48 test mosquitoes plus controls), a small number of test mosquitoes from these groups were processed in separate blots (8 and 5 mosquitoes from each time point for groups 2 and 4, respectively), the results of which are shown in Supplementary Figure 1. Cut-off values for the determination of positive and negative specimens in each run of the experiment were determined as the mean plus two times the standard deviation of the uninfected mosquito band intensities, as described previously [27].

ELISA

i) 3SP2 mAb: Anti-Pf CSP mAb 3SP2 was generated at Radboud UMC, Nijmegen, The Netherlands as described previously [14, 26].

ii) CSP-ELISA: CSP ELISA was performed as previously described [11]. Mosquitoes were homogenized in 250 µl phosphate buffered saline (PBS pH 7.2) solution with 1% sarcosil. Sterilin ELISA plates were coated with 3SP2 mAb at 5 µg/ml, diluted in PBS. Fifty µl of mosquito homogenate from all test mosquitoes was analysed in duplicate (100 µl total), alongside blank wells (50 µl of sample diluent) and a standard curve of recombinant CSP (50 µL Gennova, 0.1 µg/ml). A selection of homogenates from 15 uninfected blood-fed control mosquitoes were tested on every plate as a visual control, giving OD values between 0.06-0.08. OD values were adjusted for plate-to-plate variation by subtracting plate blank values. A universal cut-off for CSP positivity was determined at an optical density (OD) of 0.311 using maximum likelihood methods to establish CSP-negative and CSP-positive Gaussian distributions from the corrected OD values of all 336 test mosquitoes. The cut-off was set as the mean OD of the CSP-negative distribution plus three standard deviations, as previously described [36, 37].

Statistical analysis

Statistical significance between ECL Slot-blot and ELISA-based prevalence estimates and those measured by microscopic detection after dissection of mosquito midguts were evaluated using the chi-square test for homogeneity. Statistical analysis was conducted in GraphPad Prism 5.0 (GraphPad Software Inc, CA, USA), and confidence intervals for prevalence estimates were generated using STATA 12 (StataCorp., TX, USA).

Results

Infection of Anopheles stephensi mosquitoes with Plasmodium falciparum gametocyte cultures

Mosquitoes were fed blood meals containing four batches of gametocytes from *P. falciparum* cultures diluted to generate varying infection rates (Table 1). Parasite burdens and overall infection prevalences were estimated after mosquito dissection and oocyst detection and counting by microscopy on a subset of 20-30 randomly sampled mosquitoes from each group on day 8 PI. Additional paired samples of 36-48 mosquitoes were then removed from each of the four groups on days 8 and 10 PI for processing and assessment in the ECL-SB and ELISA (one sample for each day and method). The mosquitoes were randomly selected from the cages fed on the same gametocyte material by the same feeders. Since different mosquitoes were used for each of the three assays, some inherent variation in mosquito infection rates was expected. Full details of sample sizes in different test groups for all assays are in Table 1. In total, 104 test mosquitoes were analysed by microscopy on day 8 PI, 336 test mosquitoes were analysed in the ECL-SB on days 8 and 10 PI, and 336 test mosquitoes were analysed in the ELISA on days 8 and 10 PI.

Oocyst detection by microscopy

Mean oocyst intensities for groups 1 through 4 were 0.5, 0.9, 6.1 and 7.7 (range 0-22), with corresponding oocyst prevalences of 20.8, 40, 70, and 93.3%, respectively, when assessed on day 8 PI (Table 1). The percentages of infected mosquitoes with only a single oocyst were 0%, 25%, 11% and 5% respectively for groups 1 through 4.

Prevalence estimation using ECL-SB

Table 1 shows that at all oocyst intensities and time points the ECL-SB accurately estimated oocyst prevalence as detected by microscopy in mosquito samples from the same feed. All prevalence estimates were made using a cut-off threshold of two standard deviations from the mean of the IOD (integrated optical density) values obtained from ECL-SB from uninfected mosquitoes, as described previously [27]. Actual IOD values vary significantly between separate assay repeats according to the relative time exposed to X-ray, but positive/negative determination was consistently robust within experiments (Figure 1).

Prevalence estimation using ELISA

In line with previous results [11], the ELISA was not sufficiently sensitive to detect CSP present in infected mosquitoes at day 8 PI, regardless of oocyst intensity (Figure 2). However, after an additional 48 hours, the ELISA accurately estimated the microscopically determined oocyst prevalence at all oocyst intensities. No significant differences were observed between prevalence estimates made by ELISA and microscopy (**Table 1**; $p>0.29$), or between ELISA and ECL-SB compared directly ($p\geq0.4$) for mosquitoes analysed at day 10 PI.

Table 1. *Plasmodium falciparum* oocyst prevalence determined by microscopy (day 8 PI) and by the detection of CSP in the ECL-SB and colorimetric ELISA in separate mosquito samples processed 8 and 10 days PI from the same four experimental feeds

Group	Day PI	Microscopy				ECL-SB				ELISA			
		Mean oocysts (range)	Prevalence %	95% CI	n/N	Prevalence %	95% CI	n/N	p	Prevalence %	95% CI	n/N	p
A	8	0.5 (2-4)	**20.8**	7.1-42.2	5/24	**27.8**	14.2-45.2	10/36	0.54	**0.0**	0-9.7	0/36	0.00
	10	-	**-**	-	-	**25.0**	12.1-42.2	9/36	0.71	**33.3**	18.6-51	12/36	0.29
B	8	0.9 (1-3)	**40.0**	19.1-63.9	8/20	**37.5**	24-52.6	18/48	0.85	**0.0**	0-7.4	0/48	0.00
	10	-	**-**	-	-	**39.6**	25.8-54.7	19/48	0.97	**43.8**	29.5-58.8	21/48	0.78
C	8	6.1 (1-22)	**70.0**	50.6-85.3	21/30	**75.0**	57.8-87.9	27/36	0.65	**11.1**	3.1-26.1	4/36	0.00
	10	-	**-**	-	-	**80.6**	64-91.8	29/36	0.32	**75.0**	57.8-87.9	27/36	0.65
D	8	7.7 (1-17)	**93.3**	77.9-99.2	28/30	**91.7**	80-97.7	44/48	0.79	**14.6**	6.1-27.8	7/48	0.00
	10	-	**-**	-	-	**87.5**	74.8-95.3	42/48	0.41	**87.5**	74.8-95.3	42/48	0.41

Group Group of mosquitoes fed on the same gametocyte culture

Mean oocysts (range) Mean oocysts is given as the mean of all mosquitoes sampled. Range is given as the range of oocyst numbers in positive infections

Day PI Day post infection

n/N Positive mosquitoes/total mosquito sample size

p Chi-squared test p-value for homogeneity between positivity rates in the ECL-SB and ELISA relative to microscopy.

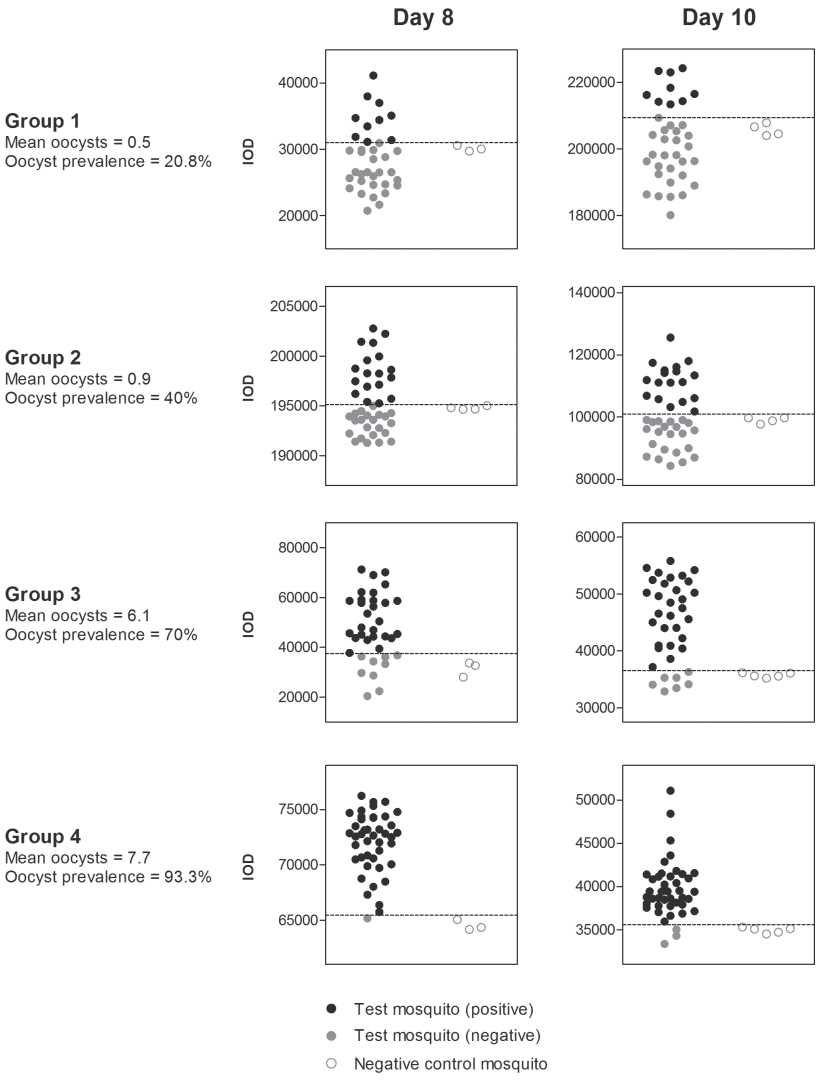


Figure 1. ECL-SB integrated optical density values for mosquito homogenate bands exposed by X-ray. The capacity of the slot blot apparatus was 48 mosquito homogenates, including three to five negative controls for the calculation of CSP positivity. Each blot was developed separately, giving rise to the varied IOD values in the figure. Positivity determination was thus blot-specific, based on the mean IOD values of the negative controls obtained in each blot. As the total number of mosquitoes processed at each time point in groups 2 and 4 exceeded the capacity of a single blot (48 test mosquitoes plus controls), a small number of test mosquitoes from these groups were processed in separate blots (eight and five mosquitoes from each time point for groups 2 and 4, respectively), the results of which are shown in Supplementary Figure 1. For clarity, the primary figure shows blots containing the majority of mosquitoes from all groups and time points.

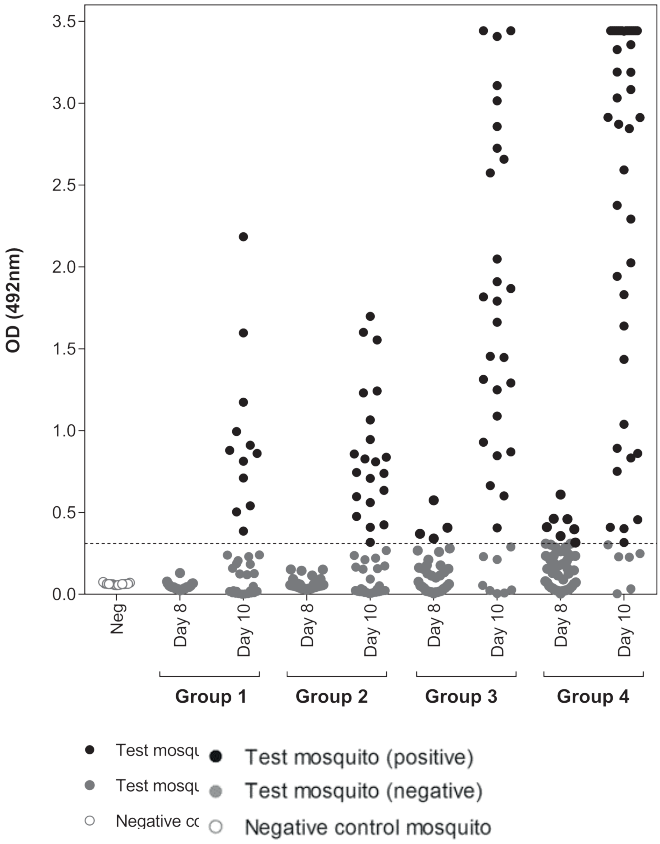


Figure 2. ELISA OD values for all test and negative control mosquitoes. Plate-specific correction of OD values against background reactivity allowed for the calculation of a universal cut-off for positivity, which was determined statistically (OD 0.311, shown as the dotted line) [36, 37, 43].

Discussion

This study describes the ability of two immuno-assays, the CSP-ELISA and ECL-SB, to detect low-density mosquito-stage *P. falciparum* infections based on the detection of CSP in the mosquito carcass. The results suggest that when mosquitoes are assayed ten days after ingestion of an infective blood meal containing gametocytes, infection detection by the CSP-ELISA and ECL-SB provide prevalence estimates that are comparable to those obtained by microscopy. If mosquitoes are assayed earlier (8 days PI), only the ECL-SB has the sensitivity to accurately estimate true infection prevalence, as compared to microscopy.

The results of the current study corroborate recent data indicating that the ECL-SB can detect CSP shortly after its production begins in parallel to the budding out of sporozoites in midgut oocysts [31]. Marginally higher prevalence estimates at day 10 PI compared to day 8 PI in groups 2 and 3, which contained single oocyst infections (mean oocyst intensity 0.9-6.1) suggest that some slower developing oocysts in low-intensity infections may be missed if the assay is performed early. The challenges to detecting developing oocysts on day 8 PI was more pronounced when the colorimetric ELISA was used [11]. A third method, the ECL-ELISA, was recently proposed as an enhancement of the standard colorimetric ELISA for the detection of CSP in whole homogenized mosquito samples [28]. This assay is capable of detecting an amount of CSP equivalent to that produced by 1.7 oocysts in mosquitoes processed at day 8 PI, a significant improvement on the colorimetric ELISA, although still possibly incapable of detecting single oocyst infections at this time point. Though our experiments were not powered to investigate the impact of oocyst intensity on assay sensitivity, the similar level of concordance with microscopy across the range of infection intensities we observed (1-22 oocysts) indicate that both ECL-SB and ELISA reliably detected single oocyst infections at day 10PI; the ECL-SB also detecting the vast majority at day 8 PI. ECL-SB prevalence estimates were in fact most concordant with microscopy in mosquito groups with only 1-4 oocysts (1 & 2), including group 2 where 25% of all infected mosquitoes sampled by microscopy harboured only a single oocyst. This indicates that our assays would have great utility for the detection oocysts in *Plasmodium* infected vectors from endemic areas (*e.g.* mosquitoes infected during direct mosquito feeding assays), which are commonly in the 1-5 oocyst range [29, 38].

In the context of public health, the prevalence of mosquitoes that are infectious to humans is the most relevant output for determining the efficacy of TRIs [16, 39]. Oocysts may produce many thousands of sporozoites, the majority capable of invading and establishing themselves in the salivary glands where they await egestion by the blood-feeding mosquito into the human dermis [12, 13]. Since mosquitoes egest very few sporozoites when feeding, a mosquito with any number of sporozoites is probably infectious [14, 15].

While the presence of salivary gland sporozoites marks infectiousness to humans, detecting earlier developmental stages may have significant operational benefits. For

mosquito-feeding experiments, storage time after feeding is a major concern as mosquito mortality may drastically limit sample size for analysis [11, 40, 41]. Early infection detection based on oocyst detection is reliable from day 6 PI by microscopy and, although a minority of oocysts may fail to produce sporozoites [9, 11], reliably predicts later sporozoite salivary gland infection [11]. Oocysts are thereby detectable before sporozoite proliferation, however, the routine microscopical detection of oocysts has a subjective element and requires highly trained microscopists to reliably detect low-density infections. CSP-based assays form an attractive alternative to microscopy because of CSP abundance, and specificity to the oocyst and sporozoite stages of sporogonic development [23]. Though CSP detection necessitates mosquito processing at day 8 PI or later [11, 27, 28], the fact of its detection may be a more reliable predictor of actual mosquito infectivity than the observation of oocyst capsules or presence of parasite DNA. For the assays in the current study, requirements for mosquito processing are modest, and equipment and assay methods relatively low-tech.

One element that is consistent for ELISA, ECL-SB and other immunological approaches to infection detection in whole mosquito homogenates is the method of mosquito homogenization. This has the benefit that mosquitoes may be killed in their cages by removal to -20°C freezers, then moved into sealed storage until analysis. Microscopy is constrained by strict scheduling based on the dates of experimental infections, whereas immunological and molecular assays may be separated from the schedule of feeding experiments at the convenience of the operator.

Another similarity between the ELISA and the ECL-SB is the relative low cost and opportunities to increase throughput, which is beneficial for low-resource settings and for settings where the proportion of infected mosquitoes is low. For the assays performed in this study, the estimated cost per mosquito was 0.96 USD for the ECL-SB and <0.1 USD for CSP ELISA. For the ECL-SB as performed here, 48 mosquitoes per apparatus can be assayed each day. For the ELISA, throughput is even higher and technology and equipment are routinely available in most research laboratories. For the CSP ELISA as performed here, 96 mosquitoes can be assayed per plate and ten plates can easily be processed by a single technician, with assays being completed in a two-day period.

Conclusions

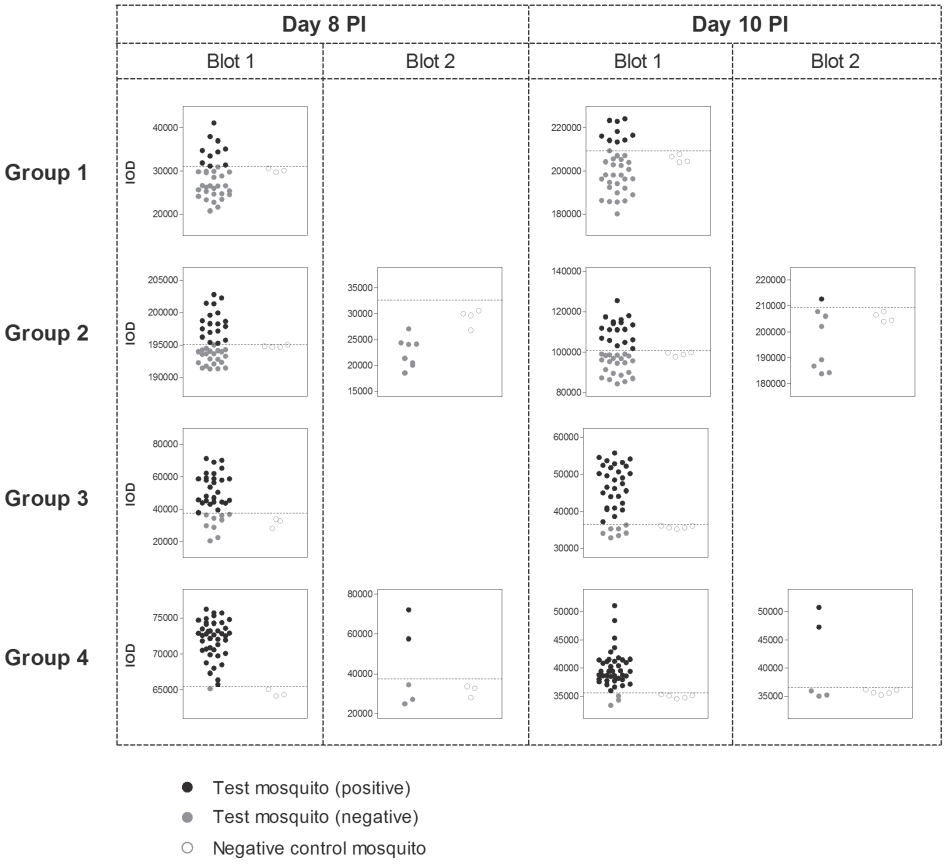
The results of this and previous studies indicate that either the ECL-SB or ELISA may replace microscopy for infection detection at day 10 PI, and that ECL-SB may do so two days earlier with no risk to assay sensitivity. Samples were not tested at day 9 PI, but it is possible the ELISA may have performed better at this time point. Availability of novel early midgut stage and trans-midgut stage, non-CSP based biomarkers may further improve the sensitivity of immunological assays for *Plasmodium* detection in mosquitoes. Efforts to develop such assays for trans-midgut stage (days 2-10 PI) *Plasmodium* detection are underway. The ECL-SB's throughput is dependent on the number of apparatuses available, while the

ELISA's throughput is essentially only limited by the number of available technicians. The remaining bottleneck to the employment of either technique for tertiary evaluation of TRI is therefore mosquito processing [42]. A reliable method of high throughput mosquito homogenization involving minimal equipment costs will further enhance the scalability of the assays described here.

Acknowledgments

We would like to thank Jacqueline Kuhnen, Laura Pelser-Posthumus, Astrid Pouwelsen, and Jolanda Klaassen for conducting all mosquito breeding and for their assistance in the evaluation of mosquito membrane-feeding experiments. WS and TB are supported by the PATH Malaria Vaccine Initiative (MVI) and by a Marie Curie Career Integration Grant from the European Community's Seventh Framework Programme (SIGNAL, PCIG-GA-2012-333936). SK was in part funded by a research grant from the PATH-Malaria Vaccine Initiative.

Supplementary figure



Supplemental figure S1. ECL-SB integrated optical density values for mosquito homogenate bands exposed by x-ray. Mosquitoes processed in separate blots but belonging to the same groups, for which cut-offs are different from the majority of mosquitoes in the same groups, are shown in this figure. All other details are as for *Figure 1*.

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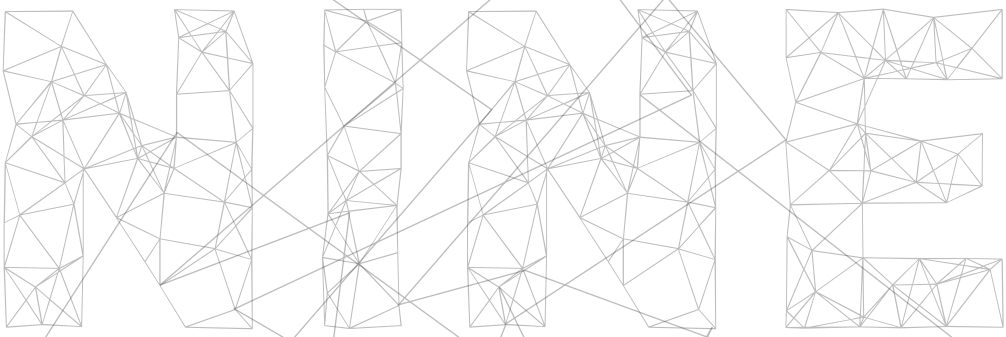
NINE

Chapter 9

Predicting mosquito infection from gametocyte density and sex-ratio

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Article type: Research Advances

This paper builds on the following paper published in eLife:
Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouedraogo AL, Basanez MG: Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection. Elife 2013, 2:e00626.

eLife (revision submitted)

Abstract

Understanding the association between gametocyte density and human-to-mosquito transmission is of immediate relevance for malaria control efforts. We related female and male gametocyte densities to the prevalence and intensity of mosquito infections in 148 feeding experiments on naturally infected gametocyte carriers from Mali, Burkina Faso and Cameroon. Male-to-female gametocyte ratio is greatest in lower density infections. The proportion of mosquitoes infected is primarily determined by female gametocyte density though statistical models indicate transmission is impeded at low gametocyte densities by a lack of male parasites. Improving the precision of gametocyte quantification simplifies the relationship between gametocytaemia and oocyst prevalence which increases rapidly before plateauing at higher densities. The mean number of oocysts per mosquito rises quickly with gametocyte density but continues to increase across the range of gametocyte densities examined. The work highlights the importance of measuring both female and male gametocyte density when estimating the human reservoir of infection.

Introduction

Mosquitoes must ingest both male and female mature gametocytes to become infected with malaria. The shape of the relationship between gametocyte density and the probability of mosquito infection is thought to be complex for *Plasmodium falciparum* [1-4]; but recent evidence has shown that the most comprehensive characterisation of this relationship was conducted using molecular tools that only quantified female gametocyte specific Pfs25 mRNA [1][5]. An additional shortcoming of these previous estimates is that female gametocytes were quantified from trendlines with unknown gametocyte sex-ratios, potentially affecting assay precision.

It is intuitive that gametocyte sex ratio is important in determining transmission efficiency [6, 7] but despite evidence that gametocyte sex ratio is adjusted in response to malaria developmental bottlenecks [6, 8, 9], there is no direct evidence for its epidemiological importance. Since mosquito infections may occur from blood with *P. falciparum* gametocyte densities below the microscopic threshold for detection [1, 10, 11] and gametocyte density itself is an important determinant of sex ratio [6, 12], sensitive quantification of male and female gametocytes is essential for assessments of the role of gametocyte sex ratio in natural infections and for reliable estimates of total gametocyte density. Here, we use a new molecular target of male *P. falciparum* gametocytes (Pf3D7_1469900 or PfMGET) [13] to explore the association between the density of female and male gametocytes and mosquito infection. The relationship between gametocyte density and oocyst intensity is also investigated for the first time as recent work has highlighted the importance of parasite intensity in mosquito-to-human transmission [14] and so both the number of infected mosquitoes and their parasite load need to be considered when assessing the human reservoir of infection.

Results

Participant characteristics

Gametocyte carriers were included from Mali (n=71), Burkina Faso (n=64) and Cameroon (n=13). *P. falciparum* parasite prevalence by microscopy ranged from ~40-70% between sites [15-18]; sampling occurred in different seasons (Table 1). All sites recruited gametocyte carriers prior to antimalarial treatment but used different enrolment criteria. Microscopically detected gametocyte carriers were included in 3 studies while one site (Balonghin) included submicroscopic gametocyte densities. Total gametocyte densities range from 0.04 to 1164 gametocytes/μL (Table 1, Figure 1). Gametocyte density estimates by microscopy and qRT-PCR were correlated in Ouelessebougou (r=0.74), Bobo Dioulasso (r=0.27), Balonghin (r=0.56) and Yaoundé (r=0.91) (Figure 1–Figure Supplement 1). Across 148 successful

membrane feeding experiments, 16.7% (1297/7757) of mosquitoes became infected, with considerable variation between gametocyte donors and study sites.

Gametocyte sex ratios in natural infections

Female gametocyte densities quantified by Pfs25 quantitative reverse-transcriptase PCR (qRT-PCR) and male gametocyte densities by PfMGET qRT-PCR were correlated (Figure 1A; Spearman's rank correlation coefficient = 0.79, $p < 0.001$). The percentage of gametocytes that were male decreased with increasing total gametocyte densities (Figure 1B, $p < 0.001$ [Kruskal Wallis test]). Conversely there was no evidence for an association between proportion of male gametocytes with asexual parasite density (Figure 1C, $p = 0.713$).

Table 1. Characteristics of gametocyte carriers and mosquito feeding assays

	Ouelessebougou, Mali	Bobo Dioulasso, Burkina Faso	Balonghin, Burkina Faso	Yaoundé, Cameroon
Number of experiments	71	19	45	13
Age, median (IRQ)	11 (7-25)	5-15 (range)*	10 (8-13)	9 (6-11)
Asexual parasite prevalence % (n/N)	64.8 (46/71)	73.7 (14/19)	73.3 (33/45)	76.9 (10/13)
Asexual parasite density per µL, median (IQR)	432 (96-2880)	360 (240-1040)	658 (336-1237)	944 (288-4224)
Gametocyte density per µL, median (IQR)	62.8 (31.4-146.8)	19.2 (10.5-26.1)	4.0 (0.6-11.0)	64.4 (11.7-126.2)
Number of mosquitoes examined per experiment, median (IQR)	70 (63-79)	29 (28-30)	40 (35-45)	37 (32-45)
Infectious individuals, % (n/N)	74.7 (53/71)	84.2 (16/19)	22.2 (10/45)	76.9 (10/13)
Infected mosquitoes, % (n/N)	17.0 (842/4960)	39.2 (208/531)	3.5 (63/1783)	38.1 (184/483)

* the age of individual gametocyte donors was not recorded in Bobo Dioulasso; gametocyte carriers were recruited from the age range 5-15 years; asexual parasite density was determined by microscopy, gametocyte density by quantitative reverse transcriptase PCR. QT-NASBA = Pfs25 mRNA quantitative nucleic acid sequence based amplification.

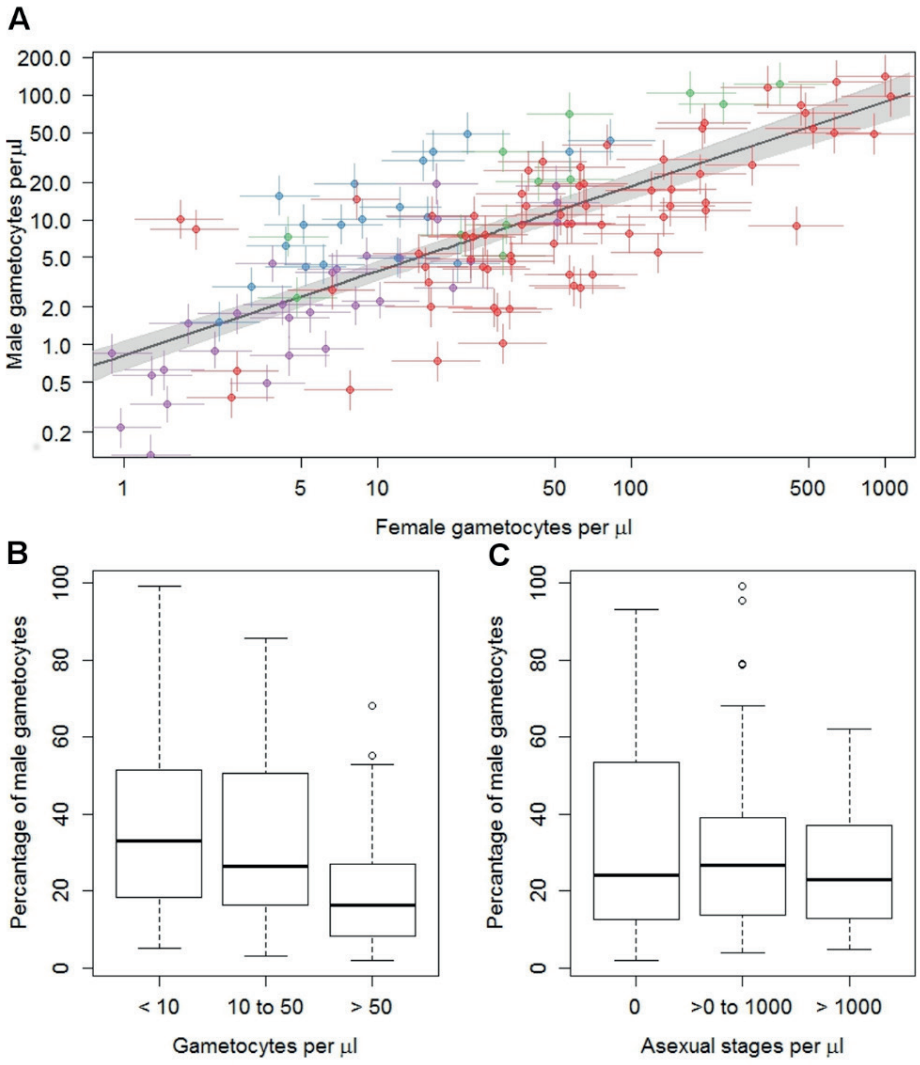

Figure 1. Gametocyte density in natural infections. The density of female gametocytes and male gametocytes is presented in panel (A) for samples from Ouelessebougou, Mali (red), Yaoundé, Cameroon (green), Bobo-Dioulasso, Burkina Faso (blue) and Balonghin, Burkina Faso (purple). Female and male gametocyte densities were positively associated ($r=0.79$, $p < 0.001$) with the best fit relationship shown by the black solid line (grey shaded area showing 95% the confidence interval around this line). Coloured horizontal and vertical lines indicate Bayesian credible intervals (CIs) around point estimates. The proportion of gametocytes that were male was negatively associated with total gametocyte density (B) but not with asexual parasite density (C). All raw data can be found in Figure 1–Source Data 1 whilst a description of the relationship modelled in (A) is provided in Figure 1–Source Data 2. The relationship between gametocyte density as measured by microscopy and PCR is given for each site in Figure 1–Figure Supplement 1.

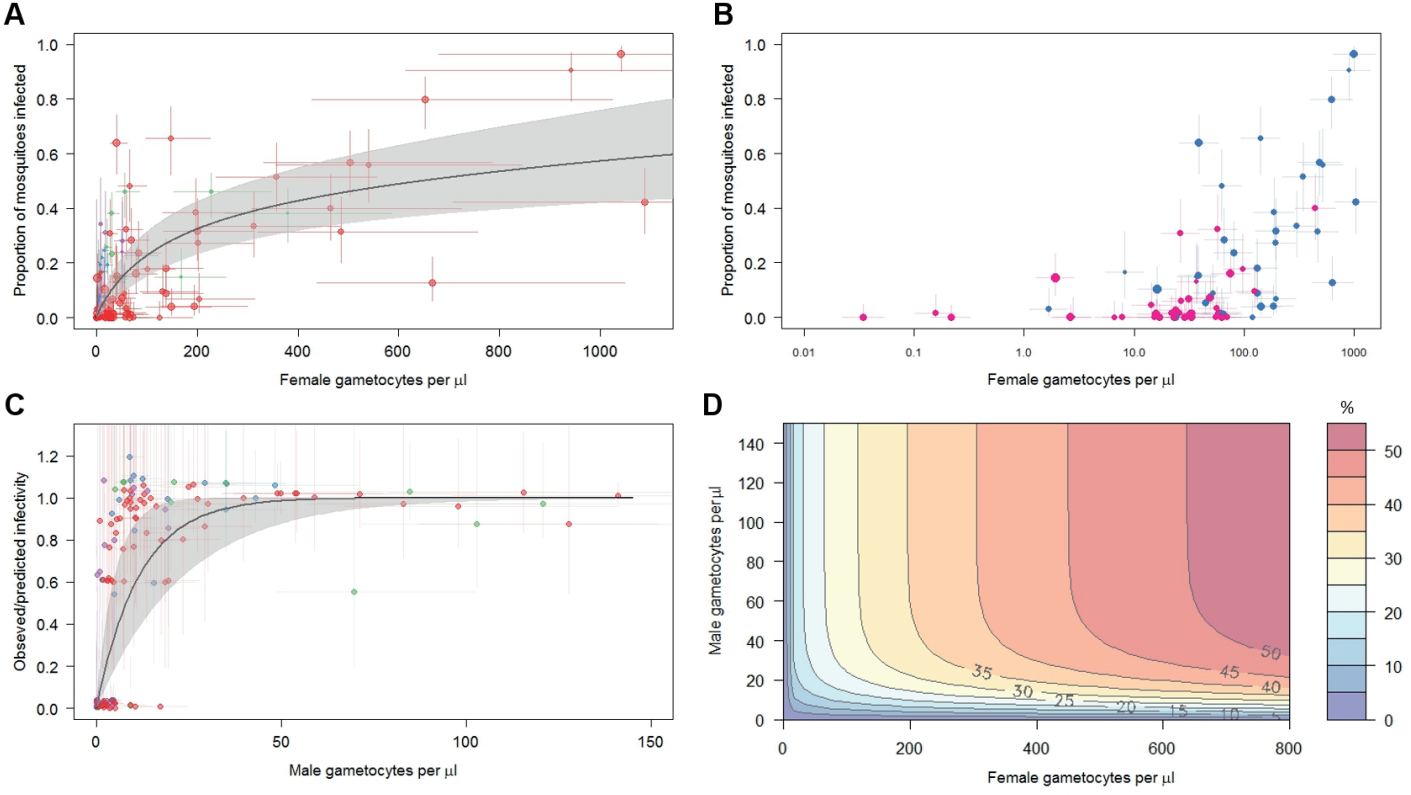
A
Proportion of mosquitoes infected
Female gametocytes per µl
B
Proportion of mosquitoes infected
Female gametocytes per µl
C
Obseved/predicted infectivity
Male gametocytes per µl
D
Male gametocytes per µl
Female gametocytes per µl
%

Figure 2. The relationship between *Plasmodium falciparum* gametocyte density and the proportion of mosquitoes that develop oocysts. **(A)** The association with female gametocyte density. The solid black line indicates the best-fit statistical model with grey shaded 95% Bayesian credible intervals (CI). Infectivity depends on both the number of male and female gametocytes so the figure uses the relationship between male and female gametocyte density defined in Figure 1A to predict overall transmission. Point colour denotes the study from which the observation came (red = Ouelessebougou, Mali, green = Yaoundé, Cameroon, blue = Bobo-Dioulasso, Burkina Faso and purple =Balonghin, Burkina Faso) and point size is proportional to the number of mosquitoes dissected. Horizontal and vertical lines indicate 95% CIs around point estimates. To aid clarity the figure shows points and model predictions scaled to the largest dataset (i.e. each site was scaled by the relative infectivity compared to the Mali dataset). A version of the figure without this scaling is shown in Figure 2–Figure Supplement 1., which shows all raw data and separate model predictions for each site. **(B)** Relationship between female gametocyte density and the proportion of infected mosquitoes for different male gametocyte densities for experiments from Mali (n=71). Points are coloured according to the number of male gametocytes (< 10 male gametocytes/µL = pink, ≥10 male gametocytes/µL = dark blue). Note bloodmeals containing lower numbers of male parasites typically have lower infectivity for a given female density. Figure 2–Figure Supplement 2 shows the same figure but differentiating between points using the sex ratio instead of absolute males density. **(C)** Model predictions for the reduction in the proportion of mosquitoes infected due to male gametocyte density. Data points are the observed infectivity divided by the predicted infectivity as predicted by the statistical model using the number of female gametocytes in the sample (colours matching panel A). Values less than one indicate reductions in relative transmission. The solid black line shows the best fit model for this restriction from the model in 2A, with shaded area and horizontal and vertical lines indicating 95% CIs.. **(D)** illustrates the best fit model predictions for the 3D relationship between female gametocyte density, male gametocyte density and the percentage of mosquitoes which develop oocysts (colour scale from 0 to ≥50% infected mosquitoes, see legend). All raw data can be found in Figure 2–Source Data 1 whilst statistical comparisons of the different curves tested in (A) are provided in Figure 2–Source Data 2.

Infectivity in relation to gametocyte density

The proportion of mosquitoes developing oocysts is best described by a model that incorporates both the number of female and the number of male gametocytes (deviance information criterion, DIC=451.5). In the best fit model female gametocyte density explains most of the variability with the proportion of mosquitoes infected increasing rapidly with increasing gametocytaemia before saturating at high female gametocyte densities (Figure 2A). At female gametocyte densities of 200 per µl approximately 40% of mosquitoes are infected. At low gametocyte densities transmission appears to be impeded by a lack of male parasites (Figure 2B). The number of mosquitoes infected is on average lower for hosts with fewer than 50 male gametocytes per µl of blood, with the model predicting that male densities <10 per µl reduces the proportion of infected mosquitoes by 50% (Figure 2C). Predictions for the proportion of mosquitoes infected according to the number of female and male gametocytes in the blood is given in Figure 2D. Poorer statistical fits were observed for statistical models where the proportion of mosquitoes with oocysts was described by either the number of females alone (DIC=481.3) or total gametocyte density (sum of male and female gametocytes, DIC=501.7). There are considerable differences in the relative infectivity between sites. Compared to the Mali data, infectivity was similar in the study in Balonghin, Burkina Faso (0.95 times as high; 95% CI 0.1 – 1.4) but 3.65 times higher in Bobo Dioulasso, Burkina Faso (95% CI 2.3 – 5.2) and 1.68 times higher in site in Yaoundé, Cameroon (95% CI 1.1 – 2.6) (Figure 2–Figure Supplement 1, Figure 2 – Source Data 2.).

Oocyst density in mosquitoes

The distribution of oocysts between mosquitoes is highly over-dispersed, with some mosquitoes harbouring very high oocyst densities. This aggregated distribution is reflected in the relationship between proportion of mosquitoes infected and mean oocyst density (Figure 3A). Mean oocyst density increased with increasing gametocyte density and continued to increase across the range of gametocyte densities observed, without evidence for a plateau being reached (Figure 3B).

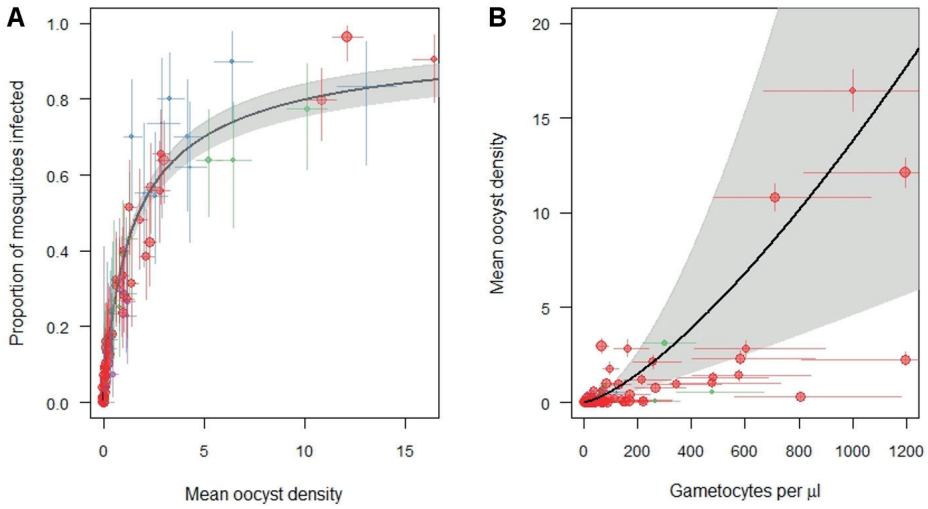

Figure 3. Associations between mean oocyst density; the proportion of mosquitoes that develop oocysts; and gametocyte density. **(A)** The relationship between mean oocyst density and the proportion of mosquitoes that develop oocysts (red = Ouelessebougou, Mali, green = Yaoundé, Cameroon, blue = Bobo-Dioulasso, Burkina Faso, purple = Balonghin, Burkina Faso; point size is proportional to the number of mosquitoes dissected). A Hill function gave the best fit to these data (DIC linear=1310; power = 602; Hill=434). **(B)** The relationship between total gametocyte density and the mean oocyst density in all mosquitoes. The power function gave the best fit to these data (DIC linear=1042; hyperbolic=1044; gompertz=1067; power=1036); total gametocyte density gave a better fit than only female gametocyte density (DIC = 1061). Horizontal and vertical lines indicate 95% Bayesian credible intervals (CIs) around point estimates, solid black line indicates the best-fit model with grey shaded area indicates the 95% CI around this line. Like Figure 2A Panel 3B aids clarify by scaling model and data to the average infectivity of the largest dataset though the raw data and model fits are provided in Figure 3–Figure Supplement 1.

Discussion

We present an improved model to predict mosquito infection from female and male gametocyte density estimates. Previous molecular assessments of *P. falciparum* gametocyte density quantified female specific Pfs25 mRNA then converted this into a measure of gametocytes per µL of blood by reference to standard curve of mixed-sex gametocytes [1, 19, 20]. These assessments therefore quantified neither female nor total gametocyte density accurately. The current model presents a considerable improvement over previous work [1], both by separately quantifying male and female gametocytes using sex-specific mRNA markers and standard curves [5] and also by using considerably more accurate estimates of total gametocyte density. Male and female gametocytes were quantified separately using automated extraction of nucleic acids [13]. Manual extraction can result in considerable

variation [22] that may have affected the accuracy of our earlier gametocyte estimates [1]. In addition, we used qRT-PCR that has higher precision than the previously used quantitative nucleic acid sequence based amplification, QT-NASBA) [21, 22].

Our analyses show that the relationship between the density of gametocytes and transmission can be adequately described by a simple saturating relationship, and that a more complicated curve with two inflection points is not needed [1]. This increase in simplicity is likely to be driven by the improved accuracy of gametocyte density estimates as models fit solely to female parasites density had a similar simplified shape. The concurrent quantification of female and male gametocytes leads to an improved estimation of total gametocyte biomass and could explain why models with information on both sexes were more accurate. However, evidence indicates that this is unlikely as the model fit to total gametocyte density (male plus female) gave a poorer fit than the model where transmission was dependent on female density unless there were very low male parasite densities. We therefore believe that this work provides direct evidence of the epidemiological importance of male gametocytes.

The work confirmed previous evidence that the proportion of male gametocytes is negatively associated with total gametocyte density [6, 12, 23], which may reflect a strategic investment in male gametocytes to maximize the likelihood of transmission in low-density infections [6, 24]. The models also indicated that male gametocyte density may become a limiting factor for transmission success at low gametocyte densities. These observations are in line with *in vitro* findings with *P. falciparum*, *P. berghei* and *P. chabaudi* where infections with a higher proportion of male gametocytes gave higher transmission success at low gametocyte densities but reduced success at higher gametocyte densities [6, 25].

The presented model considerably improved the prediction of mosquito infection rates compared to our previous manuscript [1]. Nevertheless, some level of uncertainty remains, particularly between study sites with several experiments resulting in considerably lower mosquito infection rates than predicted based on gametocyte density and sex-ratio. There are several plausible reasons for this. Naturally acquired antibody responses to gametocyte antigens may reduce transmission efficiency and form a first explanation why many mosquitoes may fail to become infected when feeding on some hosts with high-density gametocyte infections. Whilst immune responses that completely prevent mosquito infections are only sporadically detected in naturally exposed populations [26], it is plausible that functional transmission reducing immunity has reduced mosquito infection rates for a proportion of gametocyte carriers in our study. Temporal fluctuations in transmission reducing immunity [27] may also have contributed to the apparent differences in infectivity between study sites that recruited gametocyte carriers at different time-points in the season. The site with the highest infectivity in the current study (Bobo Dioulasso), recruited individuals in the dry season when the impact of transmission reducing immunity may be lowest [27]. The extent of between site heterogeneity in transmission should be

interpreted with caution since two of the four sites had <20 study participants. At all sites malaria-infected individuals were recruited prior to malaria treatment, making it unlikely that drug-induced sterilization of circulating gametocytes may have affected our analyses. Variation in the susceptibility of the mosquito colonies to parasite genotypes may provide a second plausible reason for the imperfect model fit [28]. A third hypothesis could be that our quantification of gametocytes by Pfs25 and PfMGET qRT-PCR may not fully reflect gametocyte maturity and infectivity as gametocytes may be detectable in the blood stream before [29] and after peak infectivity is reached [20]. Lastly, technical issues related to RNA degradation may affect gametocyte quantification and thus yield unreliable low gametocyte density estimates [30]. There were no apparent issues in maintaining the essential temperature control in the field, during transportation, or following RNA extraction for any of the samples included in this study, nor did the associations between microscopy and qRT-PCR gametocyte density estimates indicate (site-specific) RNA degradation that may have resulted in underestimations of true gametocyte densities.

The study shows that though the prevalence of mosquitoes with oocysts plateaus at high gametocyte densities the average number of oocysts in those mosquitoes continues to rise, as previously reported for *P. vivax* [31]. Understanding the association between gametocyte density and mosquito infection rates is of immediate relevance for malaria control efforts [2, 10]. Here we show that accurate measures of female and male gametocyte density can better predict human-to-mosquito infection, and could be used to assess the infectiousness of human populations.

Methods

Study populations and mosquito feeding experiments

Field samples were collected at four malaria endemic sites. Samples were collected prior to treatment and after written informed consent was obtained from participants or their guardian(s). Ethical clearance was provided by the National Ethics Committee of Cameroon; Ethical Review Committee of the Ministry of Health, Burkina Faso; Ethics Committee of the Malaria Research and Training Centre, Bamako; Ethics review committee Centre MURAZ; University of California, San Francisco, and London School of Hygiene & Tropical Medicine. Procedures for Ouelessebougou, Mali are described elsewhere [21]. From the trial in Ouelessebougou, baseline samples from microscopically detected gametocyte carriers were used. Additional samples were collected from asymptomatic microscopically detected gametocyte carriers aged 5-15 years in Bobo Dioulasso in Burkina Faso and Yaoundé, Cameroon. Lastly, a random selection of samples from a xenodiagnostic study in Balonghin in Burkina Faso was used [15]. Samples were eligible for selection if gametocytes were detected by Pfs25 QT-NASBA that has an estimated lower

limit of detection between 0.02-0.1 gametocytes/μL and thus provided data-points at the lower range of gametocyte densities[15]. The same membrane feeding protocol was used at all sites: local *Anopheles coluzzii* colony mosquitoes (Mali, Cameroon and Bobo Dioulasso, Burkina Faso) or colony mosquitoes comprising a mixture of *A.* coluzzii, *A. gambiae s.s.* and hybrid forms (Balonghin, Burkina Faso) were allowed to feed for 15-20 minutes on heparin blood samples until dissection in 1% mercurochrome at day 7 post-feeding and oocyst detection by two independent microscopists [32]. For all sites membrane feeding and sample collection were performed prior to antimalarial treatment

Molecular analysis of samples from naturally infected gametocyte donors

Female gametocytes were quantified by quantitative reverse transcriptase PCR (qRT-PCR) targeting female Pfs25 mRNA, as described elsewhere in detail [30] based on established protocols [33]. For male gametocytes we used a recently developed qRT-PCR [13] based on *PfMGET* (male gametocyte enriched transcript, *Pf3D7_1469900*), a transcript that is highly enriched in male *P. falciparum* gametocytes [34]. Primer sequences are provided in **Table 2**. For all qRT-PCR, mRNA was extracted from blood collected in EDTA tubes by venipuncture; 100 μL of whole blood was stored at -80°C in 500μL RNAprotect (Qiagen; for Burkina Faso and Cameroon samples) or 900 μL L6 buffer (Severn Biotech, Kidderminster, UK; for Mali samples) until automated extraction using a MagNAPure LC (Total Nucleic Acid Isolation Kit–High Performance; Roche Applied Science, Indianapolis, IN, USA). cDNA was synthesised directly from nucleic acids for the *PfMGET* assay, for which the primers are intron-spanning , and after DNase treatment (RQ1 DNase I Digest Kit, Promega) for the *Pfs25* assay, using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). qRT-PCR results were converted to male and female gametocyte densities using standard curves (ten-fold serial dilutions from 10^6 to 10 gametocytes/ml) of separate male and female gametocyte populations that were generated using a transgenic parasite line expressing a male specific fluorescence marker [13, 34]. The purity of male and female trendlines was previously confirmed by staining of sorted gametocyte populations using female gametocyte specific anti-Pfg377 antibodies [13, 35]. For both Pfs25 and PfMGET qRT-PCR, a threshold for positivity was set at 1 gametocyte per sample (0.01/μL).

Table 2. Primer sequences for the Pfs25 female marker, and male marker PfMGET.

Gene target	Forward primer	Reverse primer
Pfs25	GAAATCCCGTTTCATACGCTTG	AGTTTTAACAGGATTGCTTGTATCTAA
PFMGET	CGGTCCAAATATAAAATCCTG	GTGTTTTTAATGCTGGAGCTG

Statistical analysis

The statistical methods used here are the same at those in the original paper [1], which we briefly recapitulate here. qRT-PCR results are in the form of cycle-thresholds (TCT, which is the number of cycles it takes for the fluorescence associated with target amplification to exceed a defined threshold. The relationship between CT and gametocyte density is estimated by fitting a linear regression to CT estimates generated using a sample with known gametocyte density (a 10-fold dilution series). Let the observed CT be denoted by y then, where β_0 and β_1 are regression coefficients estimates, x is the (known) parasite density from the dilution series and ε represents a normally distributed random error ($\varepsilon \sim N(0,s^2)$).

$$y = \beta_0 + \beta_1 \ln x + \varepsilon, \quad (1)$$

Equation (1) can be rearranged to enable us to estimate gametocyte density from a CT measurement. We use a Bayesian hierarchical model to estimate the coefficients β_0 and β_1. These gametocyte density estimates are used to determine the relationship between gametocyte density and the proportion of mosquitoes developing oocysts. Four functional forms (linear, power, hyperbolic and Gompertz [1]) were each fit 3 times: on female gametocyte density alone; on total gametocyte density; and finally female gametocyte density but multiplied by a function accounting for reduced transmission at low male densities. Terms allowing for different infectivity at each site were incorporated into the models. The algebraic forms of these models are given in Figure 2-Source Data 2. The model quantifying the uncertainty in gametocyte density estimates was fit at the same time as the regression determining the relationship between gametocyte density and infectivity using Bayesian Markov Chain Monte Carlo methods assuming a Binomial error structure for each feeding experiment. Fitting the models simultaneously enabled the uncertainty in the gametocyte density estimates to be reflected in the uncertainty of the shape of the relationship. The models were compared using the DIC with a lower value indicating the most parsimonious fit.

The relationship between gametocyte density and oocyst density was examined in an analogous way, with two exceptions: 1) A negative binomial error structure was used to describe oocyst counts and 2) The increased complexity of the model precluded the inclusion of the function accounting for reduced transmission at low male densities, so each functional form was only fit twice; on female gametocyte density and total gametocyte density.

Acknowledgments

This work was supported by the Bill & Melinda Gates Foundation (AFIRM OPP1034789). TB and WS are further supported by a fellowship from the European Research Council (ERC-2014-StG 639776). The PATH Malaria Vaccine Initiative funded the collection of samples in Bobo Dioulasso, Burkina Faso and Yaoundé, Cameroon.

Supplementary materials

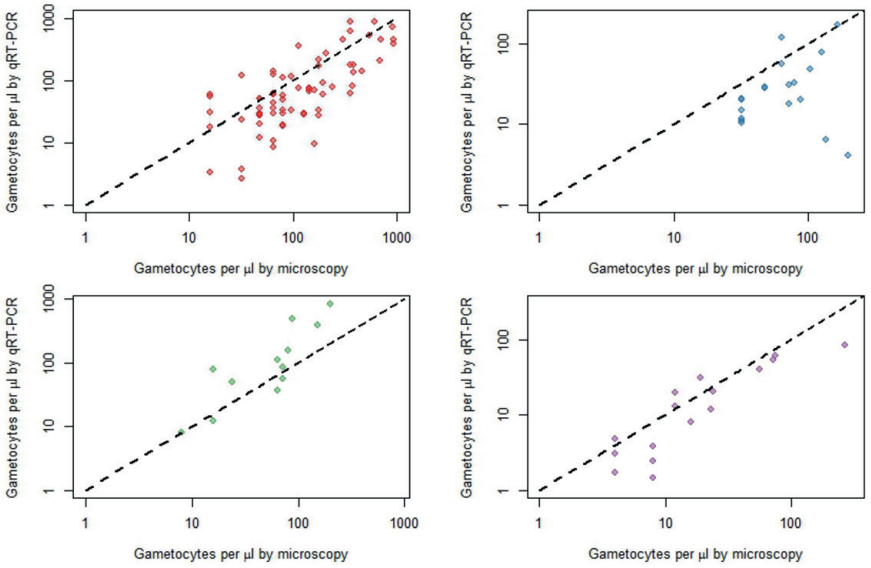

Figure 1–Figure Supplement 1. Relationship between total gametocyte densities as measured by microscopy or female gametocyte densities quantified by Pfs25 quantitative reverse-transcriptase PCR. Relationship is shown separately for each of the different sites, be it (A) Ouelessebougou, Mali (red), Yaoundé, (B) Bobo-Dioulasso, Burkina Faso (blue), (C) Cameroon (green), and (D) Balonghin, Burkina Faso (purple). Black dashed line shows the 1:1 relationship for each site.

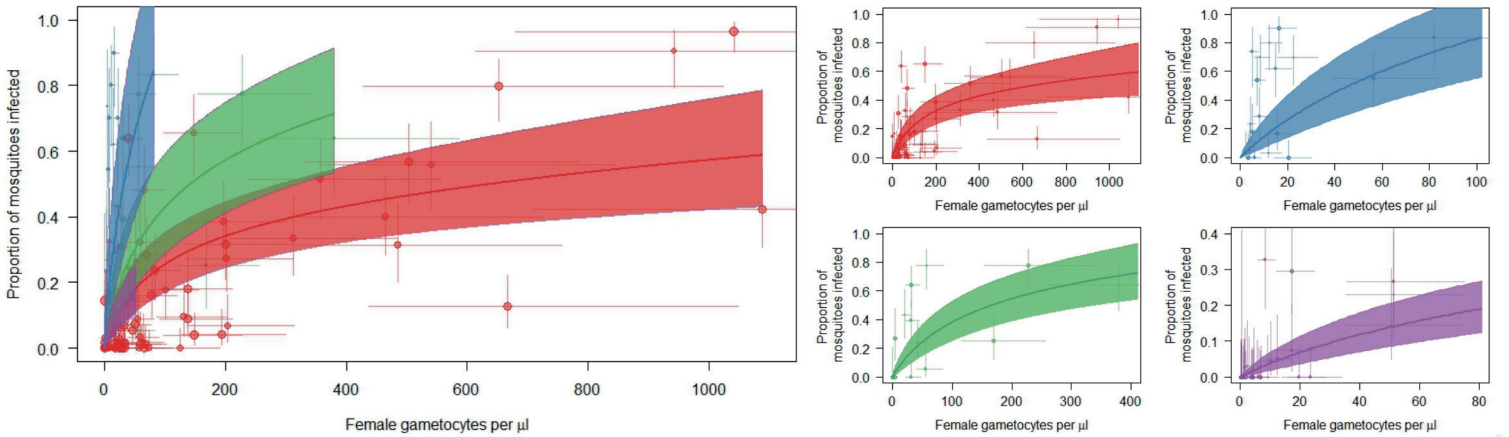

Figure 2–Figure Supplement 1. Site-specific differences in the relationship between *Plasmodium falciparum* female gametocyte density and the proportion of mosquitoes that develop oocysts. Figure is the same as Figure 2A but without the scaling the mean proportion of mosquitoes infected to the Mali dataset. Panel (A) shows all sites together whilst (B-E) show figures for each site independently. Point colour denotes the study from which the observation came: Ouelessebougou, Mali (red, B), Yaoundé, Bobo-Dioulasso, Burkina Faso (blue, C), Cameroon (green, D) and Balonghin, Burkina Faso (purple, E). Horizontal and vertical lines indicate 95% Bayesian credible intervals (CIs) around point estimates. The coloured lines indicate the best-fit model for each site with the shaded area indicates the 95% CI uncertainty around these lines. The colours of the lines correspond to the colours of the points. Best fit model predictions are projected across the gametocyte-density range observed per site.

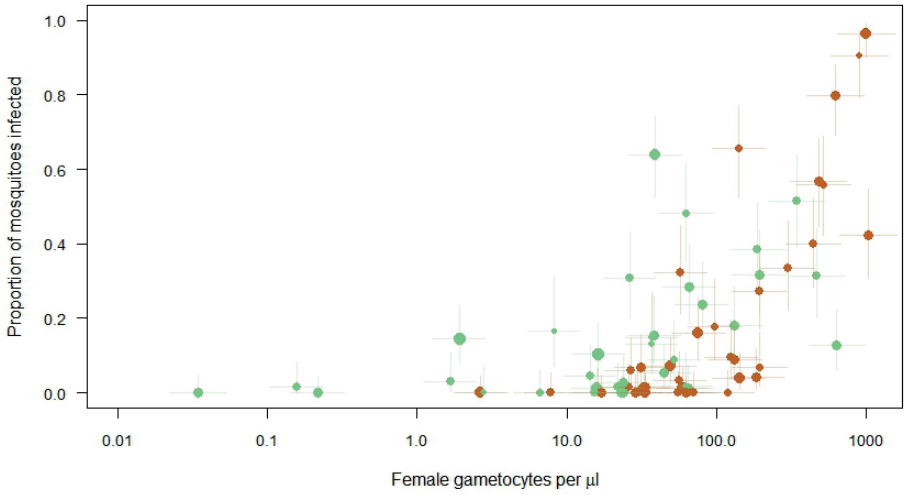

Figure 2–Figure Supplement 2. Relationship between female gametocyte density and the proportion of infected mosquitoes for different male gametocyte densities for experiments from Mali (n=71). Points are coloured according to gametocyte sex ratio: green >= 16% male, brown < 16% male (16% is the median value). At low and intermediate female densities (between 1 and 100 female gametocytes per microlitre), a high proportion of males results in higher transmission because a minimum number of males are required for successful transmission. But for higher female densities, only a small proportion of males is needed to bring the absolute number of males over the threshold for successful transmission, and beyond that extra females contribute more to infectivity.

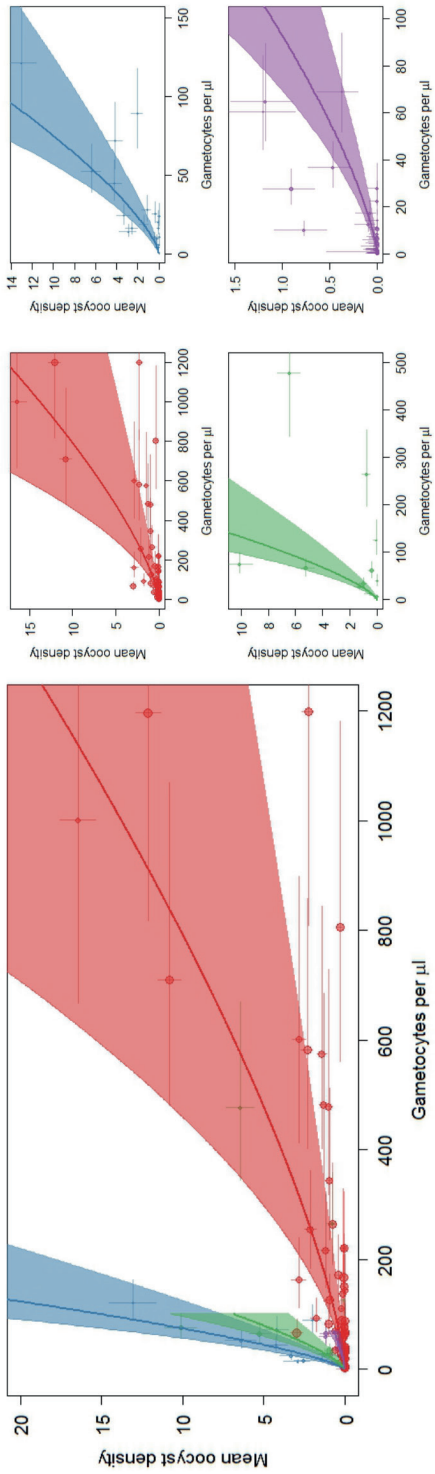

Figure 3–Figure Supplement 3. Site-specific differences in the relationship between *Plasmodium falciparum* female gametocyte density and the proportion of mosquitoes that develop oocysts. Figure is the same as Figure 3B but without the scaling average oocyst density to the Mali dataset. Panel (A) shows all sites together whilst (B-E) show figures for each site independently. Point colour denotes the study from which the observation came: Ouelessebougou, Mali (red, B), Yaoundé, Bobo-Dioulasso, Burkina Faso (blue, C), Cameroon (green, D) and Balonghin, Burkina Faso (purple, D). Horizontal and vertical lines indicate 95% Bayesian credible intervals (CIs) around point estimates. The coloured lines indicate the best-fit model for each site with the shaded area indicates the 95% CI uncertainty around these lines. The colours of the lines correspond to the colours of the points. Best fit model predictions are projected across the gametocyte-density range observed per site.

Figure 1: Source data

Least squares linear regression was used to fit a best fit line for the relationship between female gametocyte density and male gametocyte density (on the logarithmic scale).
For simplicity of presentation, the model displayed in Fig 1A is a simple linear regression on all points. The slope is 0.67 (95% CI [0.60, 0.75]) and the intercept is -0.20 (95% CI [-0.46, 0.06]) and $R^2 = 0.69$.

There is no evidence that a quadratic curve fit the data better than a linear relationship (p=0.815).

The fits for individual sites are given below:

Site	Slope [95% CI]	Intercept [95% CI]	R^2
Ouelessebougou, Mali	0.44 [0.32, 0.57]	0.56 [0.02, 1.10]	0.41
Bobo Dioulasso, Burkina Faso	0.78 [0.40, 1.15]	0.56 [-0.37, 1.48]	0.53
Balonghin, Burkina Faso	0.80 [0.67, 0.93]	-0.68 [-0.94, -0.41]	0.79
Yaoundé, Cameroon	0.82 [0.60, 1.04]	-0.02 [-0.87, 0.82]	0.86

Figure 2: Source data

Infectivity was characterised as a function of gametocyte density using the linear, power, hyperbolic and Gompertz functions described in [1]. Each curve was fit 3 times: as a function of total gametocyte density; as a function of female gametocyte density; and as a function of female and male gametocyte density (female density describing the overall shape whilst male gametocyte density allows transmission to be impeded at low densities). The full nested equation (extending the notation of the original manuscript) is given below,

$$g = [1 - \exp(-\tau \hat{x}_m')]\, f_i(\hat{x}_d')(1 + \beta_1 y_{bobo} + \beta_2 y_{bal} + \beta_3 y_{cam})$$

where $\hat{x}_d'$ is the estimated gametocyte density, be it female parasites (d=f), male parasites (d=m) or total gametocytes (sum of females and males, d=t) and τ indicates the restriction in transmission imposed by having too few males. The function $f_i(\hat{x}_d')$ determines the shape of the relationship between gametocytes and proportion of mosquitoes developing oocysts.

Subscript i indicates the functional form used, be it $f_\alpha(\hat{x}_d') = \frac{\alpha_1 \hat{x}_d'^{\alpha_2}}{1+\alpha_3 \hat{x}_d'}$ where constraining different parameters can generate a range of different shapes (linear $\alpha_2 = 1$; $\alpha_3 = 0$), power ($\alpha_3 = 0$), hyperbolic ($\alpha_1 > 0$, $\alpha_2 = 1$, $\alpha_3 > 0$) or $f_\gamma(\hat{x}_d') = \gamma_1 \exp[-\gamma_2 \exp(-\gamma_3 \hat{x}_d')]$ which generates a Gompertz (sigmoid-like) function. The variables y_{bobo}, y_{bal} and y_{cam} are indicator variables for whether a subject was from Bobo Dioulasso, Balonghin or Cameroon respectively whilst β_j is a scaling parameter between sites, be it Bobo Dioulasso (j=1), Balonghin (j=2) or Cameroon (j=3).

All models were fit to data provided in Figure 2 – Source Data 1 using methods presented in (1) which use the dilution series trendlines to convert PCR C_T values (the time taken for florescence to reach a threshold value) which is used to estimate gametocyte density. A full list of the models tested and their fit to data are provided below.

	Model fit (Deviance information criteria, DIC)		
	Total gametocyte density (*d=t*, τ =1000)	**Female gametocyte density (*d=f*, τ =1000)**	**Female and male gametocyte density (*d=f*)**
Linear ($f_\alpha(\hat{x}_d'), \alpha_2=1, \alpha_3=0$)	576.0	574.4	566.4
Power ($f_\alpha(\hat{x}_d'), \alpha_3=0$)	501.7	481.3	**451.5**
Hyperbolic ($f_\alpha(\hat{x}_d'), \alpha_2=1$)	573.0	564.8	+
Gompertz ($f_\gamma(\hat{x}_d')$)	557.4	555.7	548.3

+ models with this combination of functions failed to converge and so were discounted.

The model with the lowest DIC is the power function as a function of female gametocyte density multiplied by (1-exp(-a*g_m)) is presented in Figure 2A. Estimates and credible intervals for the coefficients are given in the table below:

Coefficient	**Estimate**	**95% credible interval**
τ	0.08573	(0.04229, 0.1967)
α_1	0.07108	(0.02295, 0.1746)
α_2	0.3021	(0.1602, 0.4751)
θ_1	2.65	(1.273, 5.174)
θ_2	-0.04733	(-0.9017, 1.405)
θ_3	0.6757	(0.06286, 1.582)

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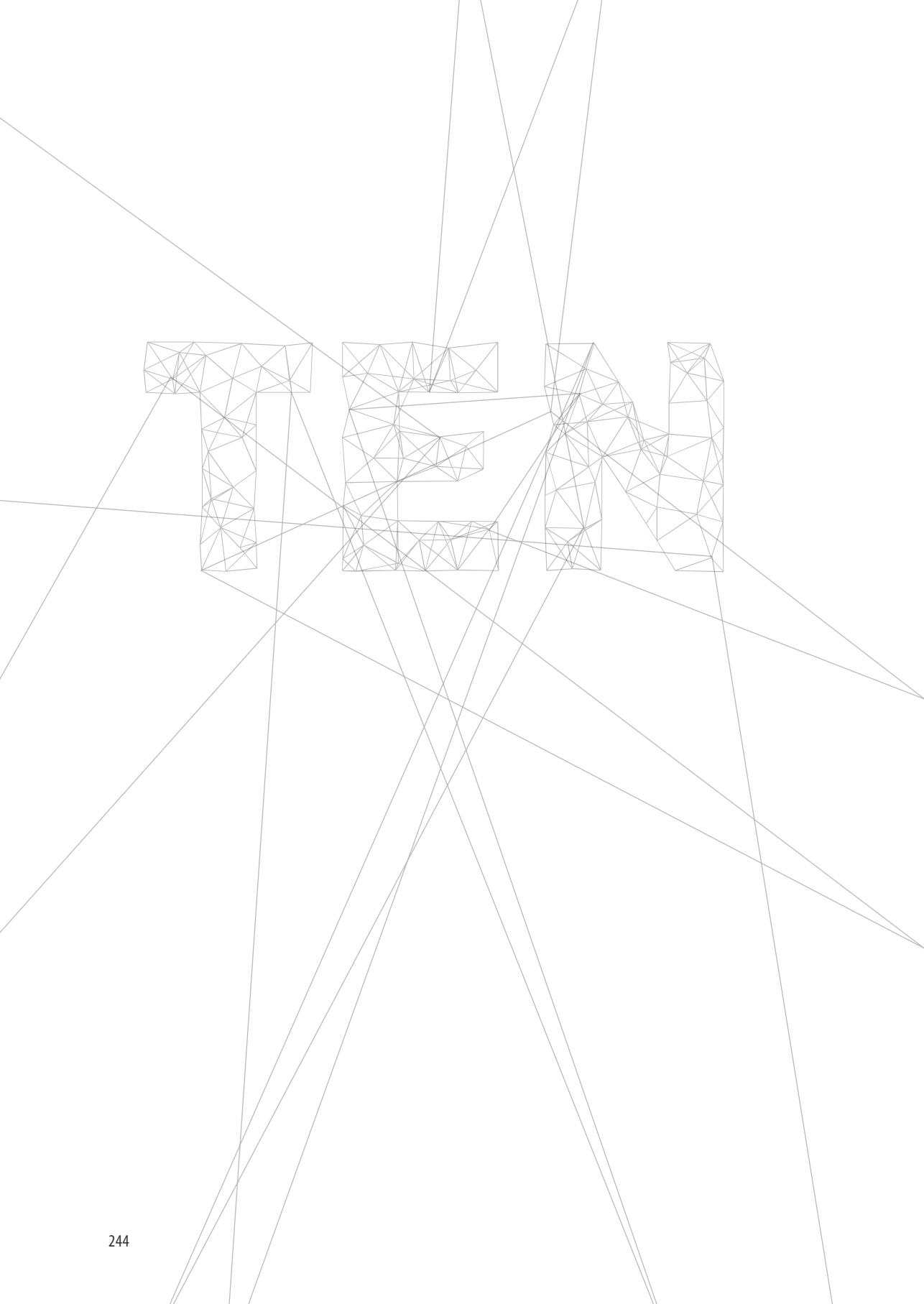
TEN

Chapter 10

A molecular assay to quantify male and female *P. falciparum* gametocytes: results from two randomised controlled trials using primaquine for gametocyte clearance

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Journal of Infectious diseases. 2016. 216:4

Abstract

Background

Single low dose primaquine (PQ) reduces *P. falciparum* infectivity before it impacts gametocyte density. Here, we examined the effect of PQ on gametocyte sex-ratio as possible explanation for this early sterilizing effect.

Methods

qRT-PCR assays were developed to quantify female gametocytes (targeting *Pfs25* mRNA) and male gametocytes (targeting *Pf3D7_1469900* mRNA) in two randomised trials in Kenya and Mali, comparing dihydroartemisinin-piperaquine (DP) alone to DP with PQ. Gametocyte sex-ratio was examined in relation to time since treatment and infectivity to mosquitoes.

Results

In Kenya, the median proportion of male gametocytes was 0.33 at baseline. Seven days after treatment, gametocyte density was significantly reduced in the DP-PQ arm relative to the DP arm (females=0.05% [IQR 0.0-0.7%] of baseline, males = 3.4% [IQR 0.4-32.9%] of baseline, $p<0.001$). Twenty-four hours after treatment, gametocyte sex-ratio became male-biased and was not significantly different between DP or DP-PQ groups. In Mali, there was no significant difference in sex-ratio between DP and DP-PQ groups (>0.125mg/kg) 48 hours after treatment, and gametocyte sex-ratio was not associated with mosquito infection rates.

Conclusions

The early sterilising effects of PQ may not be explained by the preferential clearance of male gametocytes and may be due to an effect on gametocyte fitness.

Introduction

Artemisinin combination therapies (ACT) have contributed substantially to declines in the burden of *falciparum* malaria in the last 15 years [1], due to their rapid clearance of asexual stage parasites and activity against immature gametocytes [2, 3]. ACT reduce the post-treatment transmission potential of parasites from infected humans to mosquitoes more than non-artemisinin treatments [4, 5]. However, because of their incomplete activity against mature gametocytes, patients may remain infective to mosquitoes for up to two weeks after ACT treatment [6-8]. The only currently available drug able to clear mature sexual stage malaria parasites is the 8-aminoquinoline primaquine (PQ) [9] which has been used historically for gametocyte clearance as a single dose of 0.75mg base/kg PQ in combination with a schizonticide [5, 8-12]. Concerns about haemolysis in individuals with glucose 6-dehydrogenase (G6PD) deficiency have contributed to the recent revision of the recommended dose for *P. falciparum* gametocyte clearance to 0.25mg base/kg. The WHO recommend this low dose be provided alongside standard ACT without prior G6PD status screening, for the prevention of *P. falciparum* transmission in areas with ACT resistant parasites, or areas close to elimination [13].

Though the aim of PQ treatment for *P. falciparum* is a reduction in gametocyte infectivity, there is limited direct evidence on mosquito infection prevalence in individuals treated with the currently recommended low dose [9, 14, 15]. In a recent trial where PQ was combined with a current ACT, the number of individuals infecting mosquitoes dropped from 93.3% to 6.7% over a time-window when gametocyte prevalence and density appeared unaffected by PQ [14]. This pattern appears consistent; a review of *P. falciparum* transmission after PQ treatment shows that gametocyte infectivity is generally diminished prior to observable changes in gametocyte abundance [9]. A so far untested hypothesis is that PQ may disproportionally affect male gametocytes and thus sterilize infections while gametocyte densities, largely determined by the more abundant female gametocytes [16-19], remain stable [20]. *In vitro* studies with *P. berghei* show that male gametocytes are more sensitive to a range of antimalarials [21], but PQ requires bio-activation in the liver so *in vitro* studies are not possible. Since post-treatment gametocyte densities are commonly below the microscopic threshold for detection, molecular sex-specific gametocyte assays are required to test this hypothesis in clinical trials. The only previously published male gametocyte marker is insufficiently sensitive to quantify low-density male gametocytes [19].

Here, we present results from two randomised, controlled trials of PQ in combination with dihydroartemisinin-piperaquine (DP), conducted in Kenya and Mali. Whilst this is the first report on the trial in Kenya, gametocyte dynamics and transmission data (but not gametocyte sex-ratio) were previously presented for the trial in Mali [14]. In this report, we assess the effect of PQ on gametocyte sex-ratio using quantitative reverse-transcriptase PCR (qRT-PCR) with sex-specific RNA markers [19], including a highly sensitive novel

male marker selected using sex-specific *P. falciparum* transcriptomic data (*Pf3D7_1469900*) [22]. Our analysis allowed the first direct assessment of the effect of PQ on gametocyte sex ratio and subsequent infectivity to mosquitoes.

Methods

Study design and participants

The Kenyan trial was conducted in Mbita point, western Kenya, between September 2014 and September 2015. The study area is characterised by moderate malaria transmission [23]. During visits to local schools, 5 to 15 year olds providing written consent for screening were tested for malaria infection by examination of 100 fields of a thick blood smear. Participants providing written informed consent were eligible if they were patent gametocyte carriers (1 gametocyte per 500 WBC in a thick film), 5-15 years of age, with *P. falciparum* mono-infection. Exclusion criteria were haemoglobin density (Hb) of <9.5g/dL, asexual parasite density of >200,000 parasites/μL, body mass index (BMI) of below 16 kg/m^2 or above 32kg/m^2, tympanic temperature of >39°C, antimalarial treatment taken within 2 days, recent treatment with drugs known to be metabolised by the P450 (CYP) 2D enzyme family, history of adverse reaction to study drugs, blood transfusion within 90 days, history or symptoms of chronic illness, or family history of any condition associated with extended QTc interval. The trial protocol received ethical approval from the Kenya Medical Research Institute Ethics Review Committee (#439), and the London School of Hygiene and Tropical Medicine Observational/ Interventional Research Ethics Committee (#7323).

Procedures

All participants received a three-day course of dihydroartemisinin-piperaquine (DP; Eurartesim, Sigma-Tau, Italy) alone or with a single low dose of PQ (0.25mg base/kg) on the third day of DP treatment (day 2 of the trial). Enrolees and trial staff other than the trial pharmacist, who was involved only in randomisation and drug administration, were blinded to treatment arm allocation.

PQ (Sanofi-Aventis U.S. LLC Bridgewater) was prepared as previously described by dissolving crushed 1mg tablets into distilled water and mixing with a taste-masking solution to a final concentration of 0.25mg/kg child weight [8, 24]. Participants assigned to the placebo arm received the same total volume of water and masking solution.

Participants were examined at the study clinic on days 0, 1, 2, 3, 7 and 14 after enrolment. Blood samples were taken at all time points except for day 1, either by fingerpick (day 0, day 2 and day 14) or venipunture (day 3 and day 7). At all sampling points haemoglobin levels were quantified by Hemocue photometer (HemoCue AB, Sweden) and asexual parasites and gametocytes were enumerated by examination of a giemsa stained thick film counting

against 200 or 500 white blood cells (WBC), respectively, and converted to densities per μL assuming 8000 WBC/μL. Two assays were used for gametocyte detection: quantitative nucleic acid-based sequence amplification (QT-NASBA) was used to determine gametocyte prevalence at enrolment and d2, d3, d7 and d14 following treatment; quantitative reverse-transcriptase PCR (qRT-PCR) was used for gametocyte quantification [25] and sex-ratio.

To validate our sex-specific qRT-PCR assays, we first confirmed the ability to generate populations of male and female gametocytes using a recently published PfDynGFP/P47mCherry reporter line (supplemental **Figures S1-S3**). Then, we developed and optimised sex-specific qRT-PCR assays amplifying mRNA specific to the *Pfs25* (female marker) and *Pfs230p* (male marker) genes [19] and more sensitive male targets (supplemental **Table S1**). *Pfs230p* also showed limited sensitivity in our preliminary analyses (**Supplemental tables S2-S3**), and in our baseline surveys (gametocytes were undetectable in 9/112 of baseline samples using Pfs230p). We thus designed a new qRT-PCR based on a more abundant gene transcript specific to male gametocytes*; Pf3D7_1469900* (hereafter *PfMGET* for male gametocyte enriched transcript) [22]. In the supplemental information, we provide details of target selection (**Supplemental table S2**), the development and validation of the *PfMGET* qRT-PCR assay (**Supplemental tables S3-S6**) and details on molecular gametocyte assay methodology for the Kenya and Mali trials. *Pfs25* and *PfMGET* assays showed similar sensitivity, and a threshold for positivity was set at 1 gametocyte per sample (0.002/μL) for both assays.

At day 3 and day 7 blood was provided to mosquitoes for direct membrane feeding assays, as previously described [26]. Low infectivity (38 oocyst positive mosquitoes out of 8686 dissected, from 2 individuals in the DP arm) prompted additional feeds (n=32) to be conducted with serum replacement at d0, d3 and day 7. Mosquitoes remained uninfected in these conditions. Later it was discovered that the *A. gambiae s.s.* colony was infected with Microsporidia spp. which have been shown to inhibit *Plasmodium* survival in mosquitoes [27] and precluded meaningful assessments of infectivity in this trial.

To test our findings in an independent dataset, and explore the relationship between sex ratio and infectivity, we performed a subsidiary analysis of blood samples from participants of a single blind, dose-ranging, randomised trial of DP with PQ conducted near Ouelessebougou, Mali [14]. PQ was provided with the first dose of DP at baseline, at doses of 0.0625mg/kg (n=16), 0.125mg/kg (n=16), 0.25mg/kg (n=15), and 0.5mg/kg (n=17) (control with DP only, n=16). The main outcomes of this trial were previously reported [14]. For the current study, blood samples were available from baseline and from 2, 3, 7 and 14 days after the administration of PQ. Direct membrane feeding assays for the assessment of infectivity to *Anopheles gambiae* mosquitoes were successfully performed at baseline and at days 2 and 7 [14, 26].

Sample size calculations

Sample size calculations were based on the anticipated primary outcome; the prevalence of infectivity to mosquitoes in membrane feeding assays. A previous study in the same setting showed that 30% of sub-microscopic gametocyte carriers and 80% of patent gametocyte carriers infected mosquitoes 7 days after DP treatment [7]. Assuming a conservative estimate of 30% infection after DP and assuming PQ would reduce this to below 5% [28], a sample size of 60 participants per treatment arm, allowing for 10% loss to follow up, was considered sufficient to detect this difference in infection rate with 90% power at a significance level of 0.05.

Data Analysis

The primary efficacy endpoint for the Kenyan trial was the mean gametocyte clearance time (GCT; i.e. number of days until gametocytes become undetectable by QT-NASBA [24] in the DP-PQ arm compared to the DP arm), calculated using previously presented mathematical models that allow clearance time to be extrapolated beyond the period of follow-up [29]. Secondary endpoints were the area under the curve (AUC) of QT-NASBA based gametocyte density over time (gametocytes/μL^{-1} days) [5] using log^{10} gametocyte density in linear regression models with adjustment for baseline gametocyte density, and qRT-PCR gametocyte prevalence, density, and sex ratio (proportion of total gametocytes that were male) at days 3 and 7. Stata 12.0 (Stata Corporation, College Station, TX, USA) and SAS 9.3 (SAS Institute, Cary, NC, USA) were used for statistical analysis. Baseline measures were compared between treatment arms using student's t-test, Wilcoxon rank-sum tests, or chi-squared tests. Differences between treatment arms in gametocyte prevalence and (log^{10} transformed) density after baseline were assessed with linear and logistic models, after adjusting for gametocyte density at baseline. Differences in the proportion of male gametocytes between treatment arms were compared with Wilcoxon rank-sum tests at baseline, and linear models adjusted for baseline gametocyte density after treatment. Proportions within treatment arms were compared to paired baseline measures with Wilcoxon signed-rank tests. The accuracy of gametocyte sex ratio estimates depends on the total number of gametocytes detected. To ensure robust sex ratio estimates, ratio analyses were restricted to samples with total qRT-PCR estimated counts of >16 gametocytes per sample [30], resulting in minimum gametocyte/μL thresholds of 0.032 for Kenyan samples, and 0.32 for Malian samples.

Results

5525 children were screened, and 120 gametocyte carriers were enrolled into the Kenyan trial (**figure 1**). 60 were assigned to receive DP with a placebo, and 60 were assigned to receive DP-PQ. Four participants in the DP arm and 2 in the DP-PQ arm were lost to follow up or excluded from the trial. The lowest Hb concentration recorded during the trial was 6.9g/dL, observed in one individual prior to PQ administration who was excluded from the trial. Minimum recorded Hb in all other participants was 8.6g/dL.

Gametocyte density at baseline was not significantly different between treatment arms by microscopy (p=0.705), or qRT-PCR (females, p=0.692; males, p=0.784; combined males and females, p=0.823) (**table 1**). The median proportion of male gametocytes at baseline was 0.33 (IQR 0.22-0.49) in the DP arm, and 0.32 (IQR 0.17-0.53) in the DP-PQ arm (p=0.547); i.e. a sex ratio of approximately 1 male to 2 females (**table 2**).

Gametocytocidal efficacy of low dose PQ

QT-NASBA gametocyte prevalence on day 3 (24 hours after PQ) was 89.3% (50/56) in the DP arm and 96.6% (56/58) in the DP-PQ arm (p<0.093) (**figure 2**, **table 2**). At day 7 gametocyte prevalence by QT-NASBA was 85.7% (48/56) in the DP arm, and 37.9% (22/58) in the DP-PQ arm (p<0.001). The estimated mean time to gametocyte clearance in our selective population with high starting gametocyte densities was 49.2 days (95% confidence interval [CI] 30.6-67.9) in the DP arm and 9.8 days (95% CI 7.7-12.0) in the DP-PQ arm (p<0.001). For DP, this estimate was based on an extrapolation of QT-NASBA data beyond the duration of follow-up and should therefore be interpreted with caution.

All gametocyte density and sex-ratio estimates were based on qRT-PCR. As a consequence of the higher input material for qRT-PCR in the Kenya trial (500 µL of whole blood for extraction, eluted in 10.5 µL water) compared to QT-NASBA (50 µL of whole blood for extraction eluted in 50 µL water), qRT-PCR gametocyte prevalence was considerably higher. At day 3, total gametocyte density determined by qRT-PCR was decreased to 24.81% (IQR 9.54-48.29%) of its level at baseline in the DP arm, and 35.73% (IQR 14.39-80.82%) in the DP-PQ arm (p-value after adjustment for baseline density=0.03). By day 7, total gametocyte density was decreased to 22.31% (IQR 7.38-78.22%) of its baseline level in the DP arm, and 1.43% (IQR 0.22-8.19%) in the DP-PQ arm (p<0.001). Gametocyte area under the curve (AUC) was significantly lower in the DP-PQ arm (p=0.018).

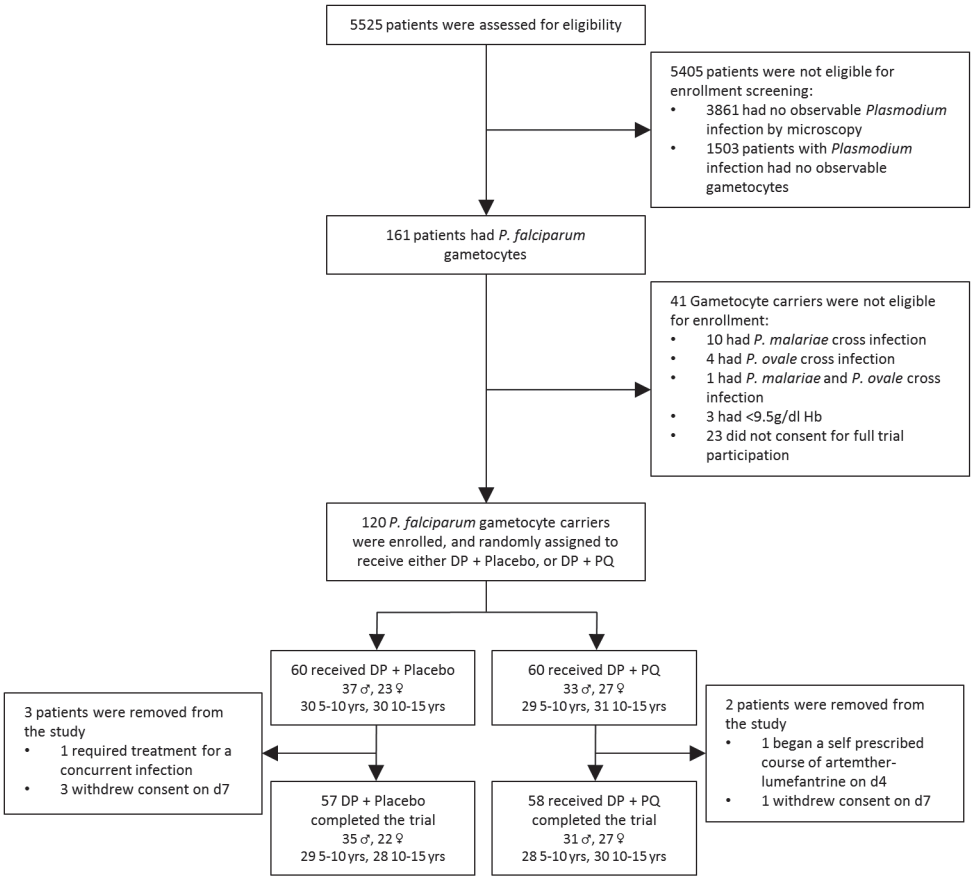

Figure 1. Screening and enrolment criteria. DP = Dihydroartemisinin piperaquine, PQ = PQ (0.25mg/kg)

Table 1: Characteristics of the study population at baseline

Characteristics at baseline	DP + Placebo (n=56)	DP + 0.25mg/kg PQ (n=58)	p value
Demographic and health			
Female % (n/N)	39.3 (22/56)	46.55 (27/58)	0.433
Age (years)	9.5 (7-11)	10 (7-12)	0.948
Haemoglobin (g/dL)	11.9 (10.7-13.0)	12.0 (11-12.4)	0.753
Microscopy			
Asexual prevalence (%)	69.09 (38/55)	59.7 (34/57)	0.297
Asexual density (parasites/µL)	360 (0-1600)	120 (0-800)	0.133
Gametocyte prevalence (%)	100.0 (55/55)	100.0 (56/56)	-
Gametocyte density (parasites/µL)	32 (16-64)	32 (16-48)	0.705
qRT-PCR			
Total gametocyte prevalence (%)	100.0 (54/54)	100.0 (57/57)	-
Total gametocyte density (parasites/µL)	10.84 (3.06-69.97)	11.87 (4.16-55.80)	0.823
Male gametocyte prevalence (%)	100.0 (54/54)	100.0 (57/57)	-
Male gametocyte density (parasites/µL)	3.76 (0.78-11.16)	3.09 (0.811-14.14)	0.784
Female gametocyte prevalence (%)	100.0 (55/55)	100.0 (57/57)	-
Female gametocyte density (parasites/µL)	6.66 (0.85-36.60)	7.64 (1.59-23.92)	0.692

Data are median (IQR) or % (positive/total sample size); - = not calculable; densities are presented for all individuals.

Effect of DP and PQ on male and female gametocytes

By day 3 female gametocyte density was decreased to a median of 9.1% (IQR 1.3-33.5%) of its baseline level in the DP arm, and 14.0% (IQR 2.9-65.2%) of its baseline level in the DP-PQ arm (p=0.152) (**table 2**, **figure 3**). Male density decreased less substantially over the same period (DP = 40.1% (IQR 15.8-86.6%) of baseline, DP-PQ= 61.8% (IQR 27.7-191.6%) of baseline, p=0.035), resulting in male biased sex ratios in both treatment arms (DP= 0.70 [IQR 0.47-0.91), DP-PQ=0.68 [0.35-0.93], p=0.625).

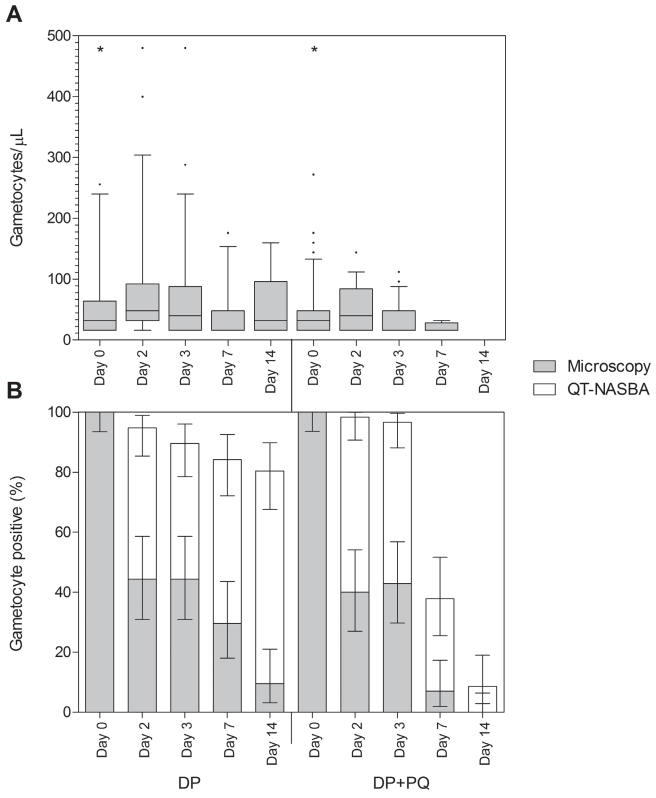

Figure 2. Gametocyte prevalence and density. A. Gametocyte density determined by microscopy at all sampling points, presented as the median, IQR and 10th-90th percentiles of gametocytes per µL for gametocyte positive individuals only. For clarity, four data points were not included in the graph; 3 in the DP only arm at day 0 (576, 608, 686/µL), 1 in the PQ arm at day 0 (1072/µL). **B.** Gametocyte prevalence determined by microscopy or *Pfs25* based QT-NASBA, presented as percent positive, with 95% CI.

At day 7, the density of gametocytes was significantly reduced in the DP-PQ arm relative to the DP arm (females=0.05% [IQR 0.0-0.7%] of baseline, males = 3.4% [IQR 0.4-32.9%] of baseline, p<0.001). In the DP arm, sex ratios were lower than at day 3 but still higher than baseline (median proportion male=0.44, IQR 0.26-0.68, p=0.002). Sex ratios among the low densities of gametocytes in the DP-PQ arm were significantly more male biased than in the DP arm (median proportion male=0.98, IQR 0.89-1.00, p values <0.001 for matched measures at baseline, and group comparison with DP arm).

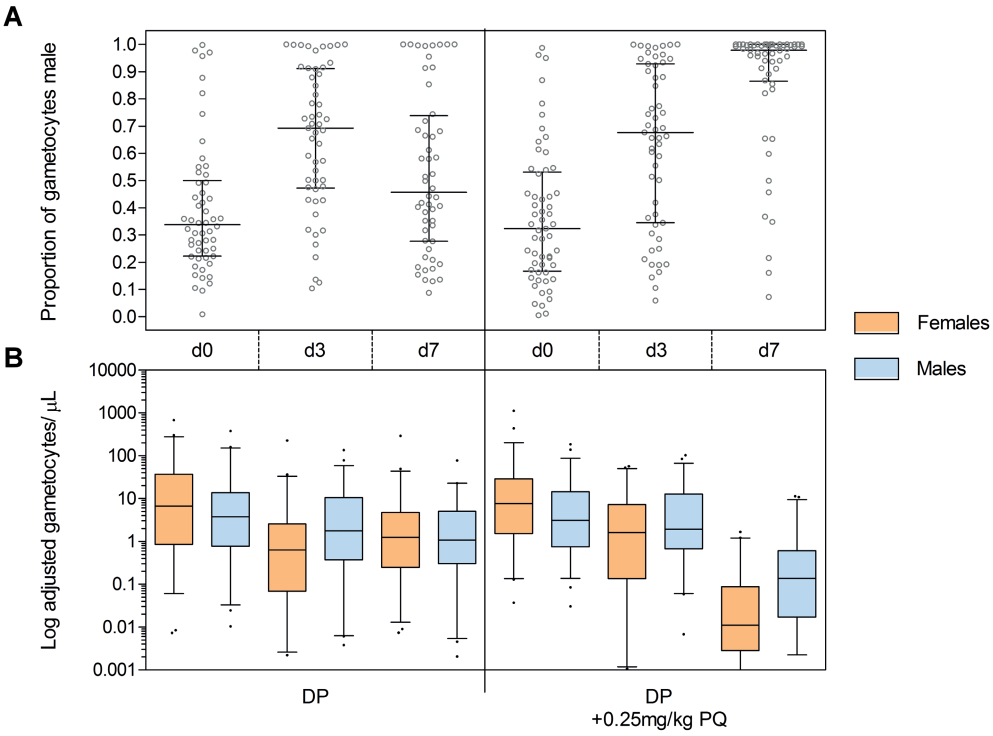

Figure 3. qRT-PCR based male and female gametocyte density and proportion male in the Kenyan study. A. Proportion of total gametocyte density that are male (males per µL/[males per µL females per µL]), presented as individual data points, with the median and interquartile range (IQR). Proportion male was only presented or included in analyses if total gametocyte density was estimated to be greater than or equal to 16 gametocytes per sample (1 gametocyte/500µL=0.032 gametocytes/µL). **B.** Gametocyte density determined by qRT-PCR at baseline (d0), and 24 (d3) or 120 (d7) hours after DP/DP-PQ. Female gametocytes were quantified by extrapolating *Pfs25* mRNA abundance from standard curves of known quantities of female gametocytes, and *vice versa*. Density is presented as median, IQR and 10th-90th percentiles of gametocytes per µL for gametocyte positive individuals only.

Effect of DP and PQ on gametocyte sex-ratio in relation to infectivity to mosquitoes in an independent trial in Mali

Pfs25 qRT-PCR based gametocyte prevalence, density, and infectivity to mosquitoes for the trial in Mali have been reported elsewhere [14]. At baseline the median proportion of male gametocytes was 0.15 (IQR 0.09-0.27) overall, and did not differ between any DP-PQ arms and the DP arm (p=0.414-0.996) (**figure 4**). At day 2 (48 hours after PQ administration) there was a significant decrease in the proportion of individuals infecting mosquitoes and the proportion of mosquitoes these individuals infected at all PQ doses of >0.125mg/kg (**figure 4**). The median proportion of gametocytes that were male at day 2 was 0.28 (IQR

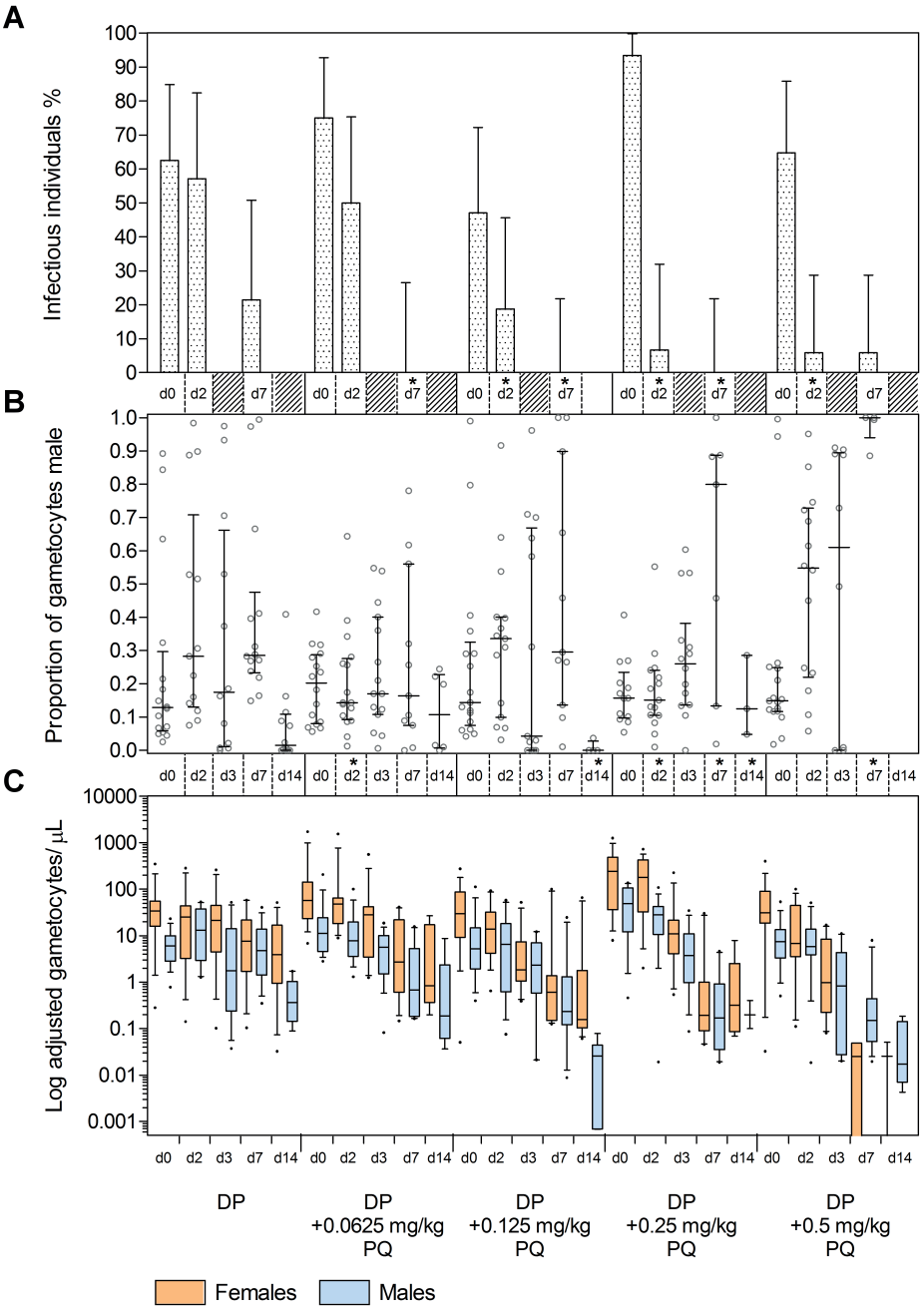
A
Infectious individuals %
B
Proportion of gametocytes male
C
Log adjusted gametocytes/ µL
DP
DP +0.0625 mg/kg PQ
DP +0.125 mg/kg PQ
DP +0.25 mg/kg PQ
DP +0.5 mg/kg PQ
Females
Males

Figure 4. Infectiousness to mosquitoes, qRT-PCR based male and female gametocyte density, and proportion male in the Malian study. A. Prevalence of infectiousness to mosquitoes among the study population in the direct membrane-feeding assay at day 0, 2 and 7. Asterisks (*) indicate significantly different infectiousness in logistic regressions relative to control. Mosquito infection was determined as the presence of any number of oocysts in the mosquito mid-gut 7 days after feeding. **B.** Proportion of total gametocyte density that are male (males per µL/[males per µL females per µL]), presented as individual data points, with the median and interquartile range (IQR). Proportion male was only presented or included in analyses if total gametocyte density was estimated to be greater than or equal to 16 gametocytes per sample (1 gametocyte/50µL=0.32 gametocytes/µL). Data from each dose arm and time point are not included in the graph if proportion male was calculable for four or less individuals per arm. Asterisks (*) indicate significantly different proportion male in logistic regressions relative to control, adjusted for baseline density **C.** Gametocyte density determined by qRT-PCR at baseline (d0), and 24 (d3) or 120 (d7) hours after DP/DP-PQ. Female gametocytes were quantified by extrapolating *Pfs25* mRNA abundance from standard curves of known quantities of female gametocytes, and *vice versa*. Density is presented as median, IQR and 10^{th}-90^{th} percentiles of gametocytes per µL for gametocyte positive individuals only. Median female gametocyte density was calculated from less than 5 individuals in some dose arms at day 7 (0.5mg/kg, n=4), and day 14 (0.5mg/kg, n=2). Median male gametocyte density was calculated from less than 5 individuals in some dose arms at day 14 (0.25mg/kg, n=3; 0.5mg/kg, n=4).

0.14-0.53) in the DP arm. Adjusted for baseline gametocyte density, the proportion of male gametocytes was borderline significantly different in the 0.0625mg/kg PQ arm (0.14 (IQR 0.09-0.27), p=0.052), but not in any higher dose PQ arms (proportion male=0.15-0.55, p=0.085-0.434). The proportion of male gametocytes was similar between DP and DP-PQ arms at day 3 (p≥0.358), but became highly male biased compared to the DP arm in the 0.25 and 0.5mg/kg DP-PQ arms by day 7 (DP=0.29 (IQR 0.24-0.41); 0.25mg/kg=0.80 (IQR 0.13-0.89), p=0.035; 0.5mg/kg=1.0 (IQR 0.99-1.0), p<0.001). Increasing PQ doses reduced the female bias of gametocytes by day 7 (**table S7**). Within each treatment arm, the proportion of male gametocytes at day 7 was significantly higher relative to baseline in the DP (p=0.016), and DP+PQ dose groups 0.125mg/kg (p=0.033) and 0.5mg/kg (p=0.043), but not in in the 0.0625mg/kg (p=0.477) or 0.25mg/kg (p=0.080) dose groups. There was no significant difference in the proportion of male gametocytes between infectious and non-infectious individuals at baseline (infectious/non-infectious = 55/26, p=0.964), at day 2 (infectious/non-infectious = 21/57, p=0.531), or at day 7 (infectious/non-infectious = 5/71, p=0.244) (**figure 5**).

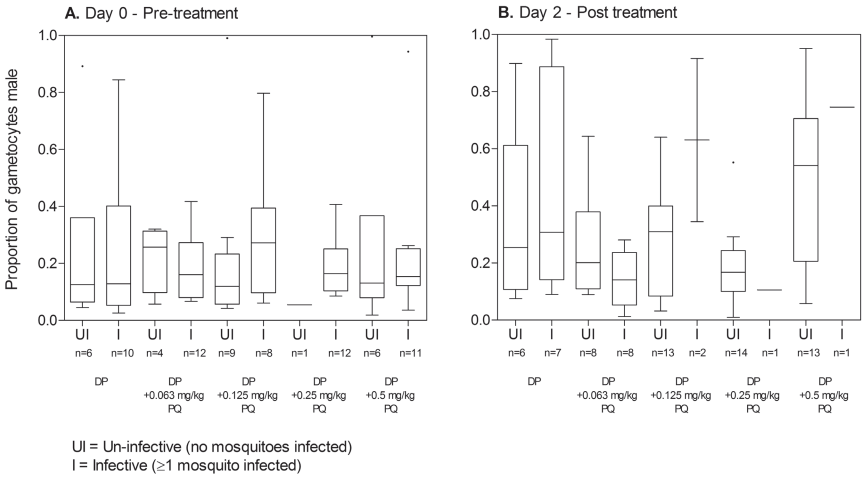

Figure 5. Proportion of male gametocytes in infectious and non-infectious samples before and after treatment in the Malian study. A. Proportion of gametocytes that were male in samples taken at baseline from individuals whose whole blood was infectious (=I) or non-infectious (=UI) to mosquitoes in the direct membrane feeding assay. Mosquito infection was determined as the presence of any number of oocysts in the mosquito mid-gut 7 days after feeding. n = number of individuals for which proportion male was calculable, and for which mosquito feeding assays were conducted. **B.** Proportion of gametocytes that were male in samples taken at day 2 (48 hours after first dose of DP in both the DP and DP-PQ arms, and only dose of primaquine in DP-PQ arms only) from individuals whose whole blood was infectious (=I) or non-infectious (=UI) to mosquitoes in the direct membrane-feeding assay. n = number of individuals for which proportion male was calculable, and for which mosquito feeding assays were conducted.

Table 2: Gametocyte measures in the Kenyan study

A. Microscopy and QT-NASBA	DP + Placebo (n=56)	DP + PQ (n=58)	p value
Gametocyte prevalence, % n/N (Microscopy)			
Day 0	100.0 (55/55)	100.0 (56/56)	-
Day 2	44.4 (24/54)	40.0 (22/55)	0.803
Day 3	44.4 (24/54)	42.9 (24/56)	0.811
Day 7	29.6 (16/54)	7.1 (4/56)	0.006
Day 14	9.6 (5/52)	0 (0/56)	-
Gametocyte prevalence, % n/N (QT-NASBA)			
Day 0	98.2 (55/56)	98.3 (57/58)	0.980
Day 2	94.6 (53/56)	98.3 (56/57)	0.334
Day 3	89.3 (50/56)	96.6 (56/58)	0.093
Day 7	85.7 (48/56)	37.9 (22/58)	<0.001
Day 14	81.8 (45/55)	8.62 (5/58)	<0.001
Gametocyte clearance time/days (95% CI)	49.2 (30.6 - 67.8)	9.8 (7.7 - 12.0)	<0.001
Gametocyte AUC/gams/μL^{-1} days (median, IQR)	55.4 (26.9-154.5)	35.4 (12.8-70.1)	0.018
B. Sex specific qRT-PCR			
Female gametocyte prevalence, % n/N (*Pfs25*)			
Day 0	100.0 (55/55)	100.0 (57/57)	-
Day 3	92.7 (50/55)	94.7 (51/57)	0.816
Day 7	88.9 (48/54)	71.4 (33/56)	0.001
Male gametocyte prevalence, % n/N (*PfMGET*)			
Day 0	100.0 (54/54)	100.0 (57/57)	-
Day 3	96.4 (54/56)	96.5 (55/57)	0.931
Day 7	96.3 (52/54)	96.5 (53/57)	0.465
Total gametocyte prevalence, % (n/N) (*Pfs25* & *PfMGET*)			
Day 0	100.0 (54/54)	100.0 (57/57)	-
Day 3	96.4 (53/55)	96.5 (55/57)	0.920
Day 7	96.3 (52/54)	96.4 (52/56)	0.933
Female gametocyte density, gams/µL (Pfs25)			
Day 0	6.650 (0.851-36.599)	7.640 (1.594-23.916)	0.692
Day 3	0.427 (0.054-2.403)	1.307 (0.111-6.277)	0.152
Day 7	0.831 (0.080-3.122)	0.003 (0.000-0.055)	<0.001
Male gametocyte density, gams/µL (*PfMGET*)			
Day 0	3.762 (0.778-11.164)	3.084 (0.8114-14.142)	0.784
Day 3	1.511 (0.303-8.854)	1.920 (0.476-12.387)	0.035
Day 7	0.870 (0.213-4.749)	0.137 (0.0140-0.600)	<0.001

Table 2: Continued

Total gametocyte density, gams/µL (Pfs25 & *PfMGET*)			
Day 0	10.836 (3.056-69.973)	11.870 (4.157-55.800)	0.823
Day 3	2.864 (0.495-14.707)	4.167 (1.128-18.080)	0.030
Day 7	2.509 (0.631-7.136)	0.140 (0.018-0.855)	<0.001
Proportion male (median, IQR)			
Day 0	0.338 (0.222-0.493)	0.323 (0.171-0.525)	0.457
Day 3	0.692 (0.475-0.910)	0.677 (0.345-0.928)	0.634
Day 7	0.457 (0.278-0.732)	0.979 (0.868-1.000)	<0.001
Proportion male (median/IQR) *≥16 gams/sample only*			
Day 0	0.332 (0.222-0.492)	0.323 (0.171-0.525)	0.457
Day 3	0.700 (0.472-0.911)	0.677 (0.345-0.928)	0.635
Day 7	0.441 (0.262-0.683)	0.977 (0.891-0.996)	<0.001

Data are median (IQR) or % (positive/total sample size), - = not calculable; densities are presented for all individuals

Discussion

Our findings support the effectiveness of a single dose of PQ (0.25mg/kg) for shortening gametocyte carriage following ACT treatment [13]. Our sex-specific qRT-PCR suggests that PQ may not preferentially clear male gametocytes. At early time points when PQ has been shown to substantially decrease infectiousness to mosquitoes (24-48 hours) [14, 20], the proportion of gametocytes that were male was comparable between DP-PQ and DP-only arms.

Before treatment, there is a non-linear relationship between gametocyte density and infectivity to mosquitoes [31-33]. After PQ treatment gametocyte density has no obvious relationship with infectivity and infections appear sterilised [14, 20, 24]. White and colleagues highlighted this phenomena in historic studies and urged that trials of PQ efficacy based on gametocyte density measures must be interpreted with caution [20]. In a recent PQ efficacy trial, it was postulated that because gametocytes were quantified using *Pfs25* based molecular assays, only female gametocytes were counted, while infections may have been sterilised by clearance of the smaller undetected male population [14]. If total clearance of one sex was the cause of PQ's rapid sterilising effects, a highly sensitive sex-specific assay would be capable of predicting post-treatment infectivity. Though we believe the assay presented in the current manuscript meets these requirements, our data indicates that PQ treatment has a similar effect to ACT treatment in terms of absolute sex ratio, in the days after treatment where infections are sterilised. The extent of the effect varied between the two trials we describe in the current study and may relate to the timing of PQ and

ACT administration, and to the limited sample size of treatment arms in the Malian trial. However, neither trial provides evidence of the hypothesised increase in female bias after PQ treatment; the Kenyan trial indicates male gametocytes may actually be cleared more slowly than females by both DP and DP-PQ. Whilst gametocyte sex-ratio is an important determinant of transmissibility and may be associated with gametocyte density, we observed no relation between post-treatment gametocyte sex-ratio and infectivity. Collection of RNA samples from a larger number of gametocyte donors participating in mosquito feeding studies conducted prior to drug administration would allow more rigorous assessment of gametocyte sex-ratio and its effect on the likelihood of onward transmission in natural infections.

Our findings do not exclude the possibility that PQ's sterilising effects are sex-specific. The *P. berghei* based *in vitro* dual gamete formation assay has shown that male gametocytes are more susceptible to a range of drugs with different modes of action [21]. The advantage of this system is that its endpoint (exflagellation in males, production of the translationally repressed Pfs25 protein in activated females) is based on gametocytes ability to activate (*i.e.* their fitness) rather than the presence of their mRNA, which may remain detectable in non-functional intact gametocytes. Quantifying gametocytes based on mRNA transcript numbers has limitations [34]. We used automated RNA extraction to minimize variation in extraction efficiency and assessed mRNA transcript numbers during gametocyte maturation. mRNA transcripts of *Pfs25* and *PfMGET* increased sharply from stage II to stage V gametocytes after which we found no evidence for age-dependent transcription patterns that may explain our findings of a faster reduction of female-specific transcripts following treatment (supplemental **figure S4**). Our findings suggest that the early sterilizing effect of PQ may be a consequence of a reduced fitness, rather than immediate clearance of one or both gametocyte sexes. Delves et al. showed that percent inhibition of activation by dihydroartemisinin was approximately 14x higher for male gametocytes than females [21]. Though this appears to conflict with our observation that male gametocytes are cleared more slowly by DP-PQ, both observations may be valid if (male) gametocytes were rendered non-functional by DP-PQ but remained in the circulation during sampling. As such, our study supports the notion that functional assays (be it gametocyte fitness or infectivity) are essential to determine the transmission-blocking properties of antimalarial drugs.

Observations of microscopy's insensitivity [6, 35-38] and the significance of sub-microscopic gametocyte densities for transmission [39-41] have placed great import on quantifying the sub-microscopic gametocyte reservoir. The recent realisation that *Pfs25* is transcribed specifically, or in far greater abundance in female gametocytes, affects previous interpretations of the *Pfs25* readout as measure of gametocyte density [19] since the male component of gametocyte biomass will have gone largely undetected and thus total gametocyte density will have been underestimated.

The results of the current study shed some light on PQ's early sterilising activity. Determining gametocyte sex-ratio may have significant utility for examining the relationship between gametocyte density, sex, and infectivity to mosquitoes, and for the assessment of drugs causing clearance of one sex. However, our findings demonstrate that the preferential clearance of one gametocyte sex does not provide an explanation for the rapid sterilizing effect of PQ. Trials assessing transmission-blocking effects of drugs like PQ should thus continue to rely on functional transmission read-outs such as mosquito feeding assays.

Acknowledgments

We would like to thank the staff of St Judes Clinic, at ICIPE, Mbita point, and particularly David Owaga, Selphas Okach, Nick Makio, George Omweri, Albert Okoth, and Addah Magawa, for their assistance with trial organisation, sample collection, and mosquito feeding. We would also like to thank Rianne Siebelink-Stoter (Radboudumc), for supportingl gametocyte culture and assisting with microscopy. We further thank Dr. Massimo Sanchez and Dr. Valentina Tirelli, (ISS) for assistance in FACS sorting. We thank Sanofi-Aventis for their kind donation of primaquine. This work was supported by the Bill and Melinda Gates Foundation (AFIRM OPP1034789); TB and WS are supported by a fellowship from the European Research Council (ERC-2014-StG 639776) and a Marie Curie Career Integration Grant from the European Community's Seventh Framework Programme (SIGNAL, PCIG-GA-2012-333936).

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Supplementary materials

A. Confirmation of sex-specificity of the transgenic PfDynGFP/P47mCherry line

The purity of male and female fractions obtained using the PfDynGFP/P47mCherry line was assessed by immune-fluorescence assays (IFA) and microscopy. This line expresses sex-specific fluorescent markers (GFP integrated under the control of the male specific *Dynein heavy chain* promoter, mCherry expression controlled by the female specific *P47* promoter in episomal plasmids), which allow separation of males and females by fluorescence-activated cell sorting (FACS) [1]. A detailed description of the development of the reporter line, culture conditions, and sorting into sex specific populations by flow cytometry has been reported [1]. For the current experiments, conducted at Istituto Superiore di Sanita in Rome, Italy, sorting was performed based on GFP signal only. On day 5 following NAG, $4x10^7$ stage III gametocytes were purified by MACS column, sorted based on GFP expression and GFP+ (male gametocytes) and GFP- fractions (female gametocytes) were brought back into separate cultures. On day 12, sorted populations had progressed to stage V gametocytes and were analysed by IFA using the gametocyte (rabbit anti-Pfg27 [2]) and female gametocyte-specific antibodies (mouse anti-Pfg377 [3] generated using immunization with recombinant Pfg377 [4]) and by microscopy examination of Giemsa stained smears. IFA analysis show that the GFP + fraction was 100% Pfg27+/Pfg377- (Figure S1, Figure S2) and are thus males. This was confirmed by examination of the Giemsa stained smears showing that gametocytes exhibit the diagnostic cytoplasmic pink staining (Figure S3). By contrast, the GFP- fraction was 83% Pfg27+/Pfg377+ and thus females (Figure S1, S2). Examination of the Giemsa stained smears confirms that the vast majority of these gametocytes exhibit the diagnostic cytoplasmic blue staining (FigureS3). A minor fraction the GFP- gametocytes (17%) are Pfg27+/Pfg377-. These could be male gcytes from a population of non-fluorescent gametocytes. These results fully support the conclusion that mCherry+/GFP- gametocytes are female gametocytes.

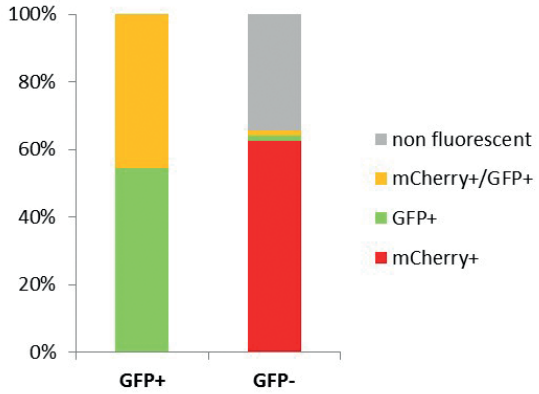
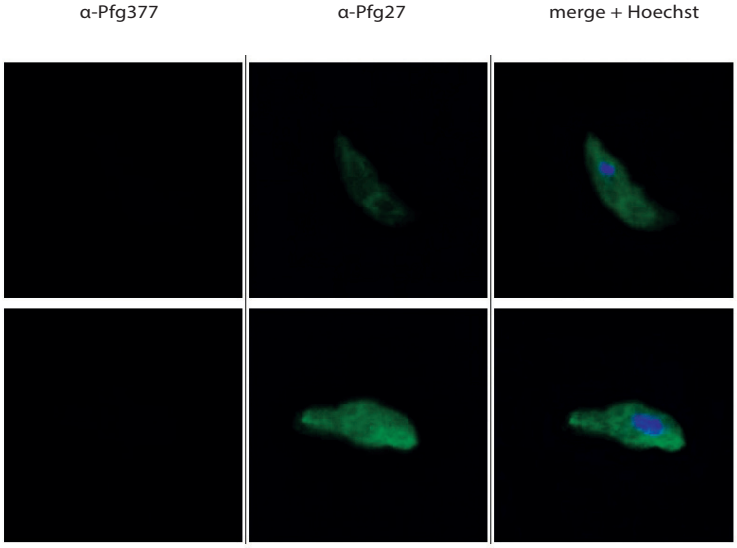


Figure S1. Counts of live fluorescent gametocytes in the GFP+ and GFP- populations and summary of IFA results. A. Counts of live fluorescent gametocytes in GFP+ and GFP- populations (based on FACS sorted parasite material).

GFP+ gametocytes (100% Pfg27+/Pfg377-)



GFP- gametocytes (83% Pfg27+/Pfg377+)

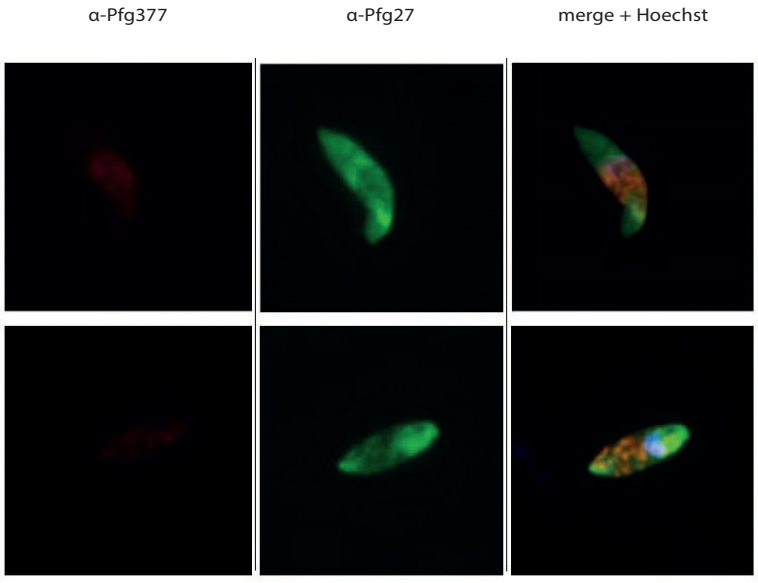


Figure S2. Representative IFA results

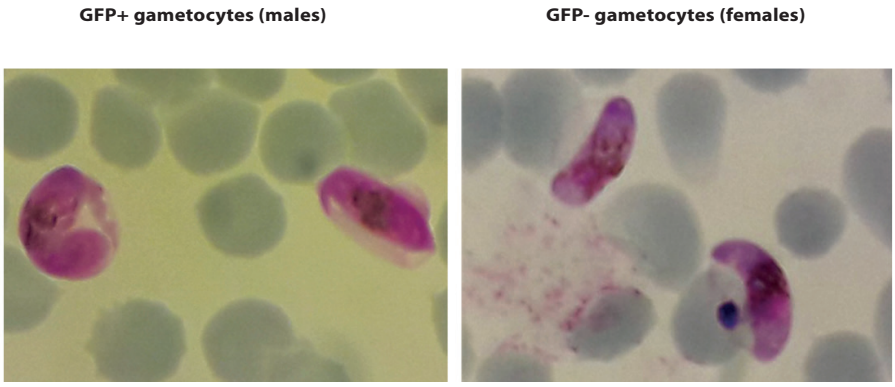


Figure S3. Giemsa staining of stage V gametocyte populations that were sorted based on GFP signal

B. Sex specific qRT-PCR development

Target selection

Sex-specific qRT-PCR assays using primers based on Pfs25 (female marker; PF3D7_1031000) and Pfs230p (male marker; PF3D7_0208900) were previously reported [5]. We tested a novel male marker, Pf3D7_1469900 (PfMGET), based on a recently published sex-partitioned transcriptomic analysis of *P. falciparum* gametocytes [1]. We selected this gene based on sex-specificity, asexual stage over gametocyte ratio, and qRT-PCR performance. **Table S1** shows the 10 genes most abundantly transcribed in male gametocytes, their abundance in female gametocytes, and their ratio in females to males. Furthermore, publically available illumina based mRNA sequence libraries (www.plasmodb.org) from seven *P. falciparum* life stages (ring, early trophozoite, late trophozoite, schizont, gametocyte stage II, gametocyte stage V, and ookinete) indicates a ratio of transcript abundance in asexual stages over mature gametocytes (in fragments per kilobase per million mapped reads (FPKM); average of all asexual stages/gametocyte stage V) of 0.36 for Pfs230p and 0.07 for PfMGET [6]. Proteins aligning to PfMGET were not observed in a recent proteomic analysis of sex separated stage V *P. falciparum* gametocytes (expressing GFP under the control of the male specific γ*tubulin II* promoter), and were detected at very low level in previous proteomic analyses [1, 7]. PfMGET was selected for further testing based on evidence of low transcription in asexual stage parasites, high transcript abundance in gametocytes, male gametocyte specificity, and on its adaptability to diagnostic qRT-PCR; PfMGET contains introns, which allowed the design of intron spanning primers (**Table S2**), thereby obviating the necessity for DNase treatment that may reduce sensitivity [8].

Table S1. Transcript levels of top 10 male genes.

Gene ID	Old Gene ID	Product	MG	FG	RATIO FG/MG
PF3D7_1201600	PFL0080c	NIMA related kinase 3 (NEK3)	17813.52	236.63	0.01
Pf3D7_1469900 (PFMGET)	**PF14_0667**	**conserved *Plasmodium* protein, unknown function**	**10110.85**	**208.93**	**0.02**
PF3D7_0406200	PFD0310w	sexual stage-specific protein precursor (Pfs16)	7991.31	10195.62	1.28
PF3D7_1413200	PF14_0131	conserved *Plasmodium* protein, unknown function	3859.07	362.29	0.09
PF3D7_0422300	PFD1050w	alpha tubulin 2	3618.96	89.36	0.02
PF3D7_1311100	PF13_0060	meiosis-specific nuclear structural protein 1, putative	2548.67	12.59	0.00
PF3D7_1105100	PF11_0062	histone H2B (H2B)	2426.04	658.45	0.27
PF3D7_0617800	PFF0860c	histone H2A (H2A)	2111.91	2573.03	1.22
PF3D7_0726000		28S ribosomal RNA	1616.88	1465.62	0.91
PF3D7_0630800	PFF1495w	conserved *Plasmodium* protein, unknown function	1541.28	235.37	0.15

Data are from Table S2 in Lasonder et al. 2016. Values for MG (male gametocytes) and FG (female gametocytes) are normalised reads per kilobase per million reads [RPKM]. Ratio is FG RPKM/MG RPKM.

Table S2. Primer sequences for the Pfs25 female marker, and male markers Pfs230P and PfMGET.

Gene target	Forward primer	Reverse primer
Pfs25 [9]	GAAATCCCGTTTCATACGCTTG	AGTTTTAACAGGATTGCTTGTATCTAA
Pfs230P [5]	CCCAACTAATCGAAGGGATGAA	AGTACGTTTAGGAGCATTTTTTGGTAA
PFMGET	CGGTCCAAATATAAAATCCTG	GTGTTTTTAATGCTGGAGCTG

For preliminary testing of the new marker, qRT-PCR assays were performed using standard curves of mixed sex NF54 gametocyte mRNA (ten-fold serial dilutions from 10^6 to 10 gametocytes/mL). Briefly, NF54 *P. falciparum* was cultured in the semi-automated culture system and started at 5% hematocrit and 0.5% parasitemia [10, 11]. Treatment with N-acetyl-glucosamine on day 7 eliminated asexual parasites, and mature stage IV/V gametocytes were harvested after 13–15 days, which was confirmed by Giemsa stained thin blood films. Gametocyte samples were serially diluted into 50µL blood, and stored in 250µL RNAprotect lysis buffer (Qiagen, Hilden, Germany). Total nucleic acid was extracted using a MagNAPure LC automatic extractor (Total Nucleic Acid Isolation Kit–High Performance; Roche Applied Science, Indianapolis, IN, USA) followed by RQ1 DNaseI digest (Promega, Sunnyvale, CA, USA) and cDNA synthesis (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). DNaseI digestion was performed for all mixed sex gametocyte samples. qRT-PCR was performed as

described previously [12], except that 1µL cDNA was used in the 20µL final reaction mix, and primer concentration was reduced to 225nM.

Despite a probable loss of sensitivity incurred to the PfMGET assay through DNaseI digestion, PfMGET mRNA appeared more abundant than Pfs230p mRNA, reflecting evidence of the transcripts relative abundance *per capita* [1, 6]. Data from assays conducted in parallel on the same material are shown in **table S3**. The lower CTs for the Pfs25 may represent higher transcript copy numbers per parasite, but are also likely the result of female bias in the mixed sex gametocyte culture.

Table S3. Pfs25, Pfs230p, and PFMGET qRT-PCR, with standard curves of mixed sex NF54 gametocytes.

Parasite/stage	Quantity (gams/mL)	Pfs25	Pfs230p	PFMGET
Gametocytes (NF54) *Mixed sex & NAG synchronised*	10^6	20.72	27.2	24.34
	10^5	23.83	29.84	27.03
	10^4	26.94	32.19	30.48
	10^3	30.14	N/A	32.82
	10^2	34.56	N/A	33.74
	10^1	34.17	N/A	N/A

Data in the table come from assays performed on the same batch of gametocyte material. The data represent means of triplicate qRT-PCR CT values, except for Pfs230 which was performed once using this material.

NAG N-acetyl-glucosamine

Gams/mL Gametocytes per mL

Gametocyte specificity

To empirically assess the specificity of PfMGET mRNA to gametocytes, and determine expression at different asexual stages, qRT-PCR assays were performed using standard curves of synchronised NF54 asexual mRNA. Asexual parasites were synchronized by the selection of late trophozoites and schizonts on a 63% percoll density gradient, followed by a 5% sorbitol treatment, killing the remaining schizonts after 5 hours, ensuring tight synchronization of parasites within a 5 hour window. Ring stage parasites were harvested 10, 20, 30 and 40 hours after percoll synchronization respectively. Parasite samples were lysed in L6 buffer and extracted immediately or frozen at -80°C until further use. Extraction and PCR conditions were the same as for the unsynchronised gametocyte samples, except that cDNA input to qRT-PCR was 2 µL.

Though input to PCR was double for the Pfs230p and PFMGET assays compared to Pfs25, transcript became undetectable at similar levels in the Pfs25 and male marker assays (threshold for detection generally >10^4) (**Table S4**).

Table S4. Standard curves of synchronised asexual stage NF54 P. falciparum.

Parasite/stage	Quantity (gams/mL)	Pfs25	Pfs230p	PFMGET
Asexuals 10hr	**10^6**	32.18	32.54	32.66
	10^5	34.91	N/A	N/A
	10^4	N/A	N/A	N/A
Asexuals 20hr	**10^6**	35.91	N/A	32.47
	10^5	N/A	37.46	34.83
	10^4	N/A	N/A	N/A
Asexuals 30hr	**10^6**	31.65	32.50	32.51
	10^5	33.51	32.89	34.23
	10^4	N/A	N/A	N/A
Asexuals 40hr	**10^6**	30.04	31.89	32.45
	10^5	34.78	N/A	N/A
	10^4	N/A	N/A	N/A

Data in the table are qRT-PCR CT values.

N/A mRNA not detectable

Gams/mL Gametocytes per mL

Male/female gametocyte specificity/sensitivity

We then used sorted male and female gametocytes to assess specificity of our assay. For this standard curves of purified female and male gametocytes (ten-fold serial dilutions from 10^6 to 10 gametocytes/mL) were constructed from gametocyte mRNA of the transgenic parasite line PfDynGFP/P47mCherry. [1]. Briefly, NF54 WT and PfDynGFP/P47mCherry parasites were cultured in the semi-automated culture system and started at 5% hematocrit and 0.5% parasitaemia [10, 11]. Treatment with N-acetyl-glucosamine (NAG) on day 7 eliminated asexual parasites to harvest mature stage IV/V gametocyte after 13–15 days, confirmed by Giemsa stained thin blood films. Gametocytes were concentrated in 37°C culture medium, separated from uninfected erythrocytes using a 63% Percoll density gradient, and subsequently taken up in a 4°C suspended activation (SA) buffer (10 mM Tris, pH 7.3, 170 mM NaCl, 10 mM glucose). Using a Coulter Epics Elite flow cytometer (Beckman Coulter) gametocytes were first separated from uninfected red blood cells using forward and sideward scatters, and then were sorted based on signal intensity of the fluorescent proteins (green fluorescent protein (GFP) or mCherry). A recent proteomic analysis of a *P. falciparum* gametocyte strain expressing GFP under the control of the male specific γ*tubulin II* promoter confirms the male specificity of Dynein heavy chain protein expression, but indicates shared expression of P47 in male and female gametocytes [13]. The observation that, upon induction of gamete formation by addition of xanthurenic acid

and a drop of temperature, exflagellation centers (i.e. male gametes) are produced only by GFP+ gametocytes and never by mCherry+/GFP- gametocytes further confirm that GFP expression is restricted to male gametocytes (Giulia Siciliano and Pietro Alano, *personal communication*).In the current study, pure male and female specific populations were obtained by excluding fluorescence negative and double positive cells. Gametocytes were FACS-sorted to obtain populations of GFP+ mCherry- (male gametocytes) and GFP- mCherry+ (female gametocytes) gametocytes, which were kept at 4°C in suspended activation (SA) buffer. Assessed by fluorescence microscopy, no GFP positive parasites were observed in the mCherry+ populations, and no mCherry positive parasites were observed in the GFP+ populations (data not shown). FACS purity analysis indicated the single fluorescent cell populations were isolated with 99.7% purity. Preliminary testing was conducted with 100 µL standard curves (**table S5**).

Table S5. qRT-PCR with Pfs25 and PfMGET and purified male and female gametocyte populations.

Quantity (gams/mL)	Batch 1		Batch 2		Batch 3			
	PfMGET	Pfs25	PfMGET	Pfs25	PfMGET	Pfs25	PfMGET	Pfs25
10^6	19.55	20.42	19.13	21.47	19.89	20.91	27.25	25.17
10^5	23.04	23.56	22.24	24.23	23.05	24.14	30.57	27.78
10^4	26.38	27.12	25.72	27.72	26.98	28.15	N/A	31.55
10^3	29.47	29.58	28.50	31.79	30.92	31.61	N/A	N/A
10^2	33.52	33.33	32.84	32.39	33.34	35.29	N/A	N/A
10^1	N/A	34.86	N/A	N/A	34.74	N/A	N/A	N/A

Males **(GFP+; mCherry-)**
Females **(GFP-; mCherry+)**

Data in the table are qRT-PCR CT values, for three biological replicates of purified male and female gametocyte material. Extraction procedures were as for the mixed sex preliminary samples, except that sample input to the magNAPure extractor was 100µL and elution was in 100µL of milliQ water.

N/A mRNA not detectable

Gams/mL Gametocytes per mL

Sex-specific gametocyte standard curves were constructed utilising the separate GFP+, mCherry- and GFP-, mCherry+ gametocyte populations. Standard curve volume in blood, storage, and extraction procedure matched those of clinical trial samples in the current study. Briefly, mRNA was extracted and eluted in 10.5µL of water using ZR Whole Blood RNA miniprep kit (Zymo Research, Irvine, CA) from blood stored at a ratio of 1 part to 3 in Blood RNA buffer (Zymo Research, Irvine, CA) at -20 °C. cDNA was synthesised (High

Capacity cDNA Reverse Transcription Kit, Applied Biosystems) directly from nucleic acids for the PFMGET assay, and after DNase treatment (RQ1 DNaseI, Promega) for the Pfs25 and Pfs230p assays. The PFMGET assay was also performed using the DNAse digested RNA, for comparison. qRT-PCR reactions were conducted as previously described [9], except that primer concentrations were reduced to 225 nM and cDNA input into the final 20 µL reaction mixture was adjusted to 2 µL. The results of testing using the optimised standard curves is shown in **Table S6.**

Table S6. qRT-PCR with Pfs25, Pfs230p and PFMGET primers using male (GFP+) and female (mCherry+) high volume gametocyte standard curves.

Extraction material	Total NA		RNA		RNA		
Marker	**PfMGET**		**P230p**	**Pfs25**	**PFMGET**	**P230p**	**Pfs25**
10^6	14.43	**16.64**	19.35	22.05	24.02	25.89	**15.53**
10^5	17.83	**19.69**	22.39	25.57	27.63	29.19	**20.02**
10^4	21.44	**23.16**	25.99	29.27	30.37	32.94	**23.13**
10^3	24.98	**26.18**	29.06	30.75	N/A	N/A	**26.26**
10^2	28.21	**29.29**	33.1	N/A	N/A	N/A	**30.08**
10^1	30.98	**33.44**	N/A	N/A	N/A	N/A	**33.44**

Quantity (gams/mL)

Males **(GFP+; mCherry-)**

Females **(GFP-; mCherry+)**

Data in the table are single qRT-PCR CT values. Sample input was 500µL. Total NA = Not DNAseI treated, RNA = DNAseI treated. Bold = Standard curve and sample preparation for analysis of Kenyan samples (for Malian samples, same preparation but lower volume – 50µL to match the sample volume).

N/A mRNA not detectable

Gams/mL Gametocytes per mL

Removing the DNAse digestion step from RNA preparation for the PfMGET assay improved the assays sensitivity notably (increase of approximately 2 CT's in all biological replicates). The Pfs25 and PfMGET assays detection limits for gametocytes of their cognate sex were <0.01 gametocytes/µL (the lowest gametocyte density used in the standard curves), while the detection limit of the Pfs230p assay for male gametocytes was closer to 0.1 gametocytes/µL. When the female and male markers were tested using standard curve material from the alternate sex, the sensitivity of the assays decreased by a factor of 100-1000 (10^3/10^4 male gametocytes were detectable using the Pfs25 assay, 10^1 were detectable using the PfMGET assay, and vice versa for female parasites). The residual Pfs25

and 146 transcript detected in standard curve of the opposite sex indicates either that both genes are transcribed in small quantities in gametocytes of the opposite sex, or that the purified standard curves were contaminated by approximately 0.1-1% of the opposite sex (in line with the purity of the sorting as assessed by FACS purity analysis). As the level of background signal was similar for both Pfs25 and 146, it was deemed unlikely to have an effect on the quantification of either sex or the determination of sex ratio in mixed sex samples. Sex ratio analyses were restricted to samples with total qRT-PCR estimated counts of >16 gametocytes per sample (gametocyte/μL thresholds of 0.032 for Kenyan samples, and 0.32 for Malian samples), to improve reliability [14].

For all clinical trial samples, separate aliquots of mRNA were processed for the Pfs25/Pfs230p assays and for the PfMGET assay, as for the standard curve material. Only samples and standard curves material retained for the Pfs25/Pfs230p assays were DNAse digested. qRT-PCR quantification was performed with matching sex standard curves to ensure that male/female specific RNA transcript abundance in field samples could be reliably converted to counts of male or female gametocytes. For the Pfs25 and PfMGET assays a cut off for positivity was set as 1 gametocyte per sample (2 gametocytes per mL/0.002 gametocytes per μL).

C. Pfs25 and PfMGET transcript abundance during gametocyte maturation

Relating gametocyte transcript numbers to gametocyte densities comes with uncertainties. One of these uncertainties is related to the stability of the number of transcripts during gametocyte maturation. Figure S5, he figure below, that is now also provided in the supporting information presents mRNA transcript counts for Pfs25 and PF_14699000 (PfMGET) in a time-course experiment. Culturing of NF54 was done without prior gametocyte sex-sorting and female (top panel) and male (bottom panel) transcripts were quantified in the same source culture material using Illumina RNAseq with paired reads, 37bp in length. Transcript counts after normalization for library size are presented for d4 till d16 after synchronization; stage IV gametocytes were observed on day 5 and stage V gametocytes on day 7. These results indicate that for both male and female markers transcript abundance is low in stage IIb gametocytes and peaks in stage V gametocytes after which transcript numbers appear stable (Pfs25) or may decline at late time-points (PfMGET). It is currently unclear whether these patterns *in vitro* may be directly extrapolated to *in vivo* transcript numbers (as is indicated in the revised discussion section). However, an increase in transcript numbers per male gametocyte appears less likely based on these data.

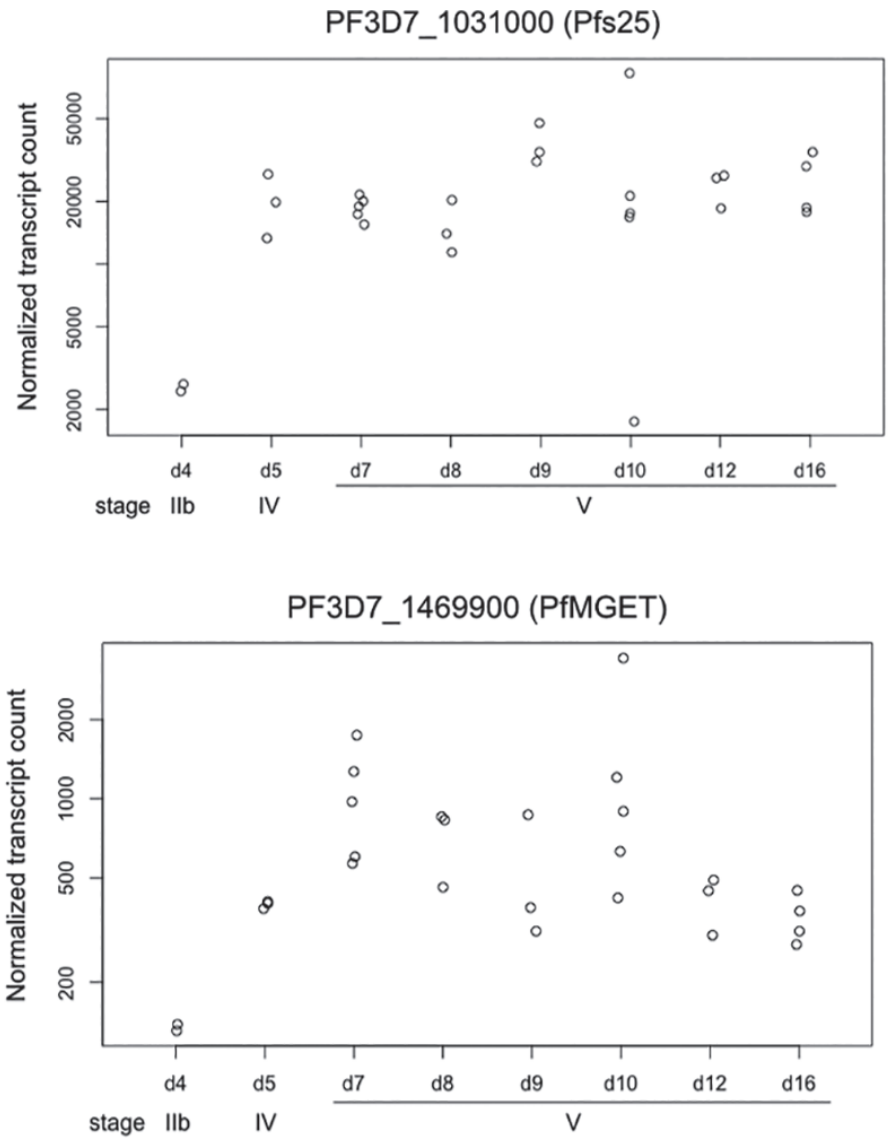


Figure S4. Transcript stability during gametocyte maturation.

D. Additional clinical trial data

Table S7. Proportion of male gametocytes in Mali by day and treatment arm.

Day	DP alone	DP+0.0625mg/kg PQ		DP+0.125mg/kg PQ		DP+0.25mg/kg PQ		DP+0.5mg/kg PQ		Dose trend
	Prop. Male (Median/IQR)	Prop. Male (Median/IQR)	P value (Coeff., SE)	Prop. Male (Median/IQR)	P value (Coeff., SE)	Prop. Male (Median/IQR)	P value (Coeff., SE)	Prop. Male (Median/IQR)	P value (Coeff., SE)	P value (Coeff., SE)
0	0.128 (0.061-0.269)	0.202 (0.082-0.285)	0.543 (-0.05,0.081)	0.144 (0.086-0.291)	0.878 (0.012,0.08)	0.157 (0.101-0.203)	0.414 (-0.071,0.086)	0.149 (0.117-0.247)	0.996 (0.000,0.08)	0.927 (0.013,0.142)
2	0.283 (0.141-0.529)	0.143 (0.096-0.271)	0.052 (-0.179,0.09)	0.335 (0.099-0.401)	0.434 (-0.071,0.091)	0.150 (0.105-0.241)	0.085 (-0.174,0.099)	0.548 (0.233-0.722)	0.291 (0.098,0.092)	0.063 (0.327,0.173)
3	0.174 (0.014-0.618)	0.170 (0.108-0.400)	0.516 (-0.08,0.122)	0.042 (0.000-0.638)	0.849 (-0.024,0.124)	0.261 (0.137-0.331)	0.761 (-0.041,0.135)	0.610 (0.000-0.891)	0.254 (0.153,0.133)	0.135 (0.358,0.236)
7	0.285 (0.238-0.411)	0.164 (0.075-0.560)	0.586 (-0.066,0.12)	0.295 (0.135-0.895)	0.599 (0.061,0.114)	0.800 (0.133-0.887)	0.035 (0.35,0.161)	1.000 (0.994-1.000)	<0.001 (0.571,0.148)	<0.001 (1.308,0.282)
14	0.014 (0.000-0.102)	0.107 (0.010-0.222)	0.272 (0.065,0.057)	0.000 (0.000-0.018)	0.322 (-0.064,0.063)	0.125 (0.048-0.285)	0.128 (0.126,0.079)			0.927 (0.013,0.142)

Prop. Male Proportion of gametocytes male

Dose trend A trend in the proportion of gametocytes that were male with increasing PQ dose was tested using a linear regression model where the DP-only arm had a value of 0 and the other arms had the value of the PQ dose (e.g. 0.0625, 0.125, 0.25, 0.5).

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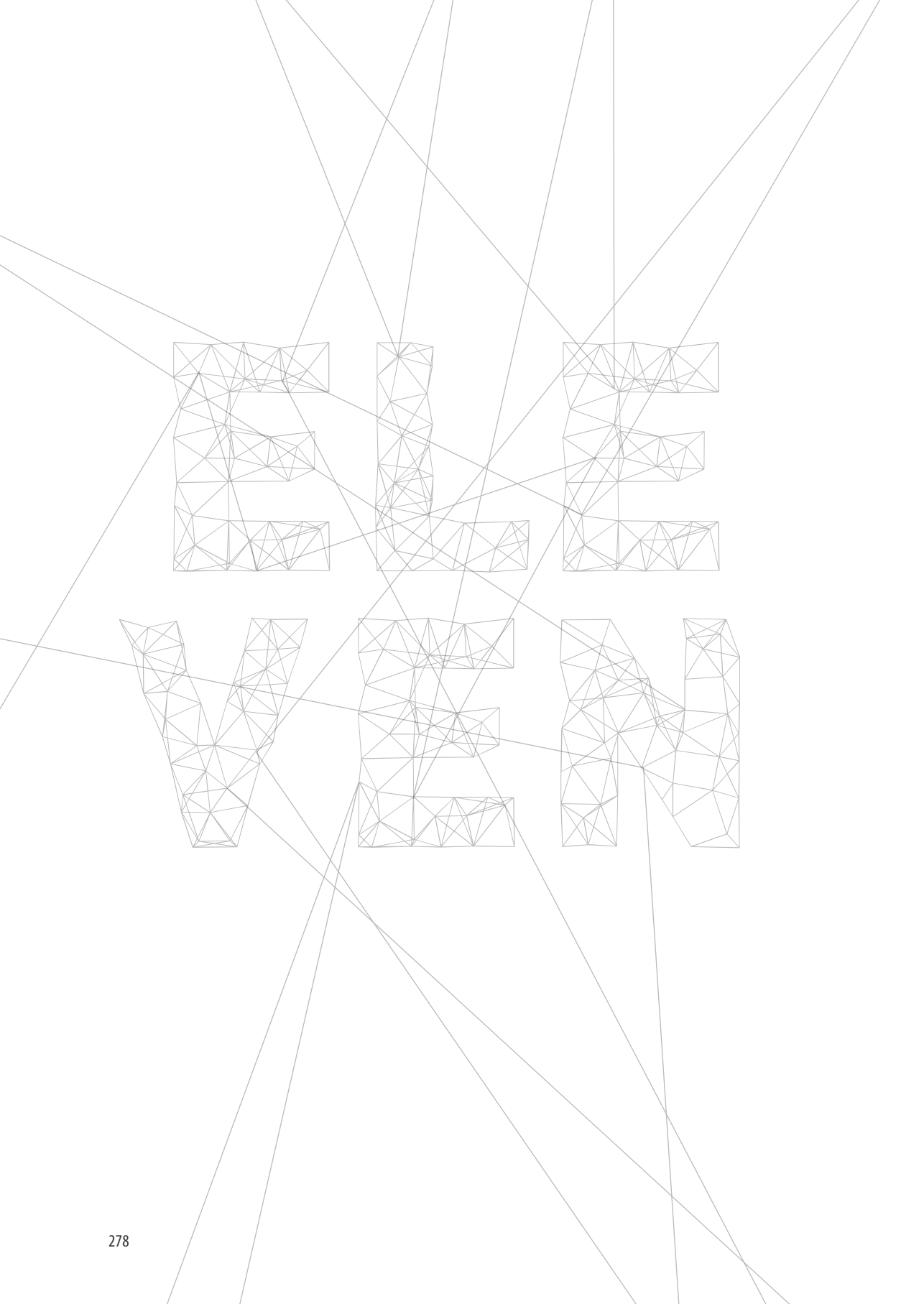
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Chapter 11

Naturally acquired immunity to sexual stage *P. falciparum* parasites

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Parasitology. 2016;143(2):187-98

Summary

Gametocytes are the specialized form of *Plasmodium* parasites that are responsible for human-to-mosquito transmission of malaria. Transmission of gametocytes is highly effective, but represents a biomass bottleneck for the parasite that has stimulated interest in strategies targeting the transmission stages separately from those responsible for clinical disease. Studying targets of naturally acquired immunity against transmission-stage parasites may reveal opportunities for novel transmission reducing interventions, particularly the development of a transmission blocking vaccine (TBV). In this review, we summarize the current knowledge on immunity against the transmission stages of *Plasmodium*. This includes immune responses against epitopes on the gametocyte-infected erythrocyte (GIE) surface during gametocyte development, as well as epitopes present upon gametocyte activation in the mosquito midgut. We present an analysis of historical data on transmission reducing immunity (TRI), as analyzed in mosquito feeding assays, and its correlation with natural recognition of sexual stage specific proteins Pfs48/45 and Pfs230. Although high antibody titers towards either one of these proteins is associated with TRI, the presence of additional, novel targets is anticipated. In conclusion, the identification of novel gametocyte-specific targets of naturally acquired immunity against different gametocyte stages could aid in the development of potential TBV targets and ultimately an effective transmission blocking approach.

Introduction

Malaria remains one of the largest global health threats, with the greatest burden of mortality and morbidity in developing countries of tropical and sub-tropical regions. Despite considerable efforts to control the disease and block its spread, 3.3 billion people remained at risk of contracting malaria in 2010, resulting in an estimated 660,000 deaths [1], primarily among young children and women in their first pregnancy. The difficulties in eliminating malaria with currently available tools [2] and the emergence of artemisinin resistance in Southeast Asia [3, 4] highlight the need for interventions that specifically aim to reduce the transmission of the parasites responsible for the disease.

Gametocytes are the highly specialized form of *Plasmodium* parasites that are infectious to mosquitoes and thus required for successful transmission from humans to mosquitoes. Their formation and infectivity to mosquitoes is presented in **Figure 1**. *P. falciparum* gametocytes progress through five distinct morphological stages, until they reach the specific elongated crescent shape characteristic of *falciparum* malaria. Male and female parasites are independently differentiated but genetically identical haploid stages that originate from asexual precursor stages; current evidence favors a model where one schizont gives rise to either male or female gametocytes [5, 6]. The only gametocytes that are observed in peripheral blood are sexually committed ring-stage parasites and fully mature stage V gametocytes [7, 8]. Intermediate stages II-IV are sequestered mainly in bone marrow compartments [9-11]. Although no genomic replication is present in gametocytes and the digestion of hemoglobin ceases after six days of development, gametocyte-specific mRNA is produced and a subset of transcripts is translationally repressed until gametocytes are taken up in the mosquito blood meal and rapidly transform into gametes [12, 13]. The reduction in temperature, rise in pH and exposure to xanthurenic acid inside the mosquito induces gametogenesis, initiating activation and expression of stage-specific proteins [14, 15]. After three rounds of DNA replication, male gametocytes exflagellate to release eight motile microgametes from the host cell that are targeted at the fertilization of female macrogametes [16, 17]. The resulting zygotes elongate to form ookinetes that traverse the mosquito midgut and develop into oocysts. Sporozoites then develop within oocysts and, approximately 10 days after the blood meal, rupture the oocyst capsule and render the mosquito infectious by invading the insect's salivary glands, completing the cycle of transmission [18].

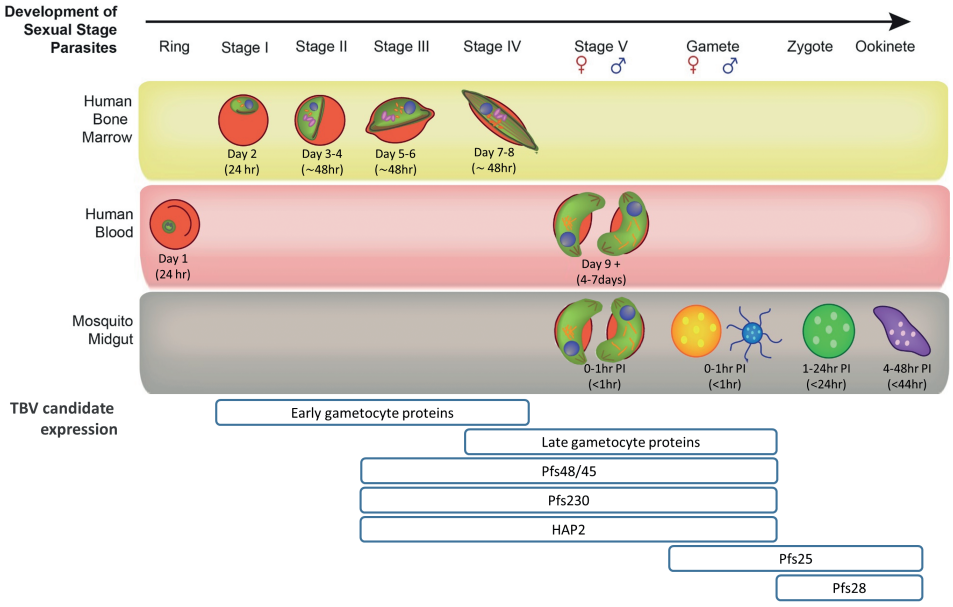

Figure 1. Maturation and location of *P. falciparum* transmission stage parasites. Timings are given cumulatively as the time since gametocyte formation, or where indicated as time post mosquito infection (PI), and as the stage-specific exposure time in brackets.

Transmission of malaria parasites from humans to mosquitoes is highly effective, with infectious humans capable of giving rise to >100 secondary human infections [19]. However, many factors influence the infectivity of gametocytes, and their transmission potential [20, 21]. Gametocyte prevalence and density are highest in infants and young children [22], decreasing with age in parallel with cumulative exposure to malaria infection and acquired immunity to the parasite's asexual stages [23, 24]. Though once thought to be rare, molecular detection methods have revealed that gametocytes are produced by the majority of infected individuals of all ages [21, 25-29] and that onward malaria transmission is not restricted to microscopically detectable gametocyte densities [30-33]. In addition to gametocyte maturity and sex ratio, human immune factors targeting the sexual stages of the parasite also highly influence transmission efficiency [21].

Here, we review the evidence for naturally occurring human immunity targeted to the transmission stages of *Plasmodium* parasites, discuss hypotheses for the mechanisms of this immunity, and suggest future research directions that may aid the design of transmission reducing interventions.

Immune responses to transmission stage parasites

Transmission Reducing Immunity (TRI) can be directed against numerous targets during gametocyte formation, maturation and transmission. Within the human host, a humoral response targeting parasite-specific epitopes on the gametocyte-infected erythrocyte (GIE) surface of different developmental stages could contribute to a decreased transmission capacity. As erythrocytes do not express Major Histocompatibility Complex (MHC) molecules and consequently lack organelles required for antigen and MHC class I complex processing, presentation to and activation of CD8+ T cells is thought to play a minor role in immune responses to the red blood cell stages of the parasite, including gametocytes [21]. Phagocytosis by monocytes and neutrophils may provide a protective immune mechanism against schizont- and merozoite-infected erythrocytes [34, 35], and though there is evidence of early stage GIE phagocytosis *in vitro* [36], evidence is limited for a functional role *in vivo* [37, 38]. Infected red blood cells may, however, be sensitive to cytokines released during general inflammatory responses. During periods of peak parasitaemia, non-specific inflammatory responses may kill both asexual blood stage parasites and circulating gametocytes, resulting in substantially reduced infectivity [39, 40]. Clinical immunity to human malaria is associated with reduced induction of cytokines and complementary parasite killing factors [40, 41], but there is no evidence that such factors have activity in the mosquito midgut. By comparison, there is substantial evidence that naturally acquired humoral responses to gametocyte proteins play a role in determining transmission efficiency.

Early stage gametocyte infected erythrocyte surface antigens

Early stage gametocytes of *P. falciparum* (stage I-IV) sequester primarily in the bone marrow [9]. Previously, it has been assumed that developing gametocytes are not recognized by the immune system, but more recent evidence provides a rationale for the existence of immunogenic proteins on the GIE surface. Sutherland *et al.* proposed the involvement of immunogenic gametocyte proteins in GIE adhesion to microvasculature, as well as spatial and temporal signal transduction, in the human host [42]. For example, parasite sensing of signals via host receptors or via non-specific permeability pathways could induce changes in the host RBC erythrocyte adhesive phenotype and thus affect the timing of gametocyte release into circulation. In a Thai cohort, naturally acquired antibody responses reduced transmission to mosquitoes and distorted the morphology and maturation of young gametocytes [43]. Some of these plasma antibodies also bound the GIE surface from gametocyte stage II onwards, suggesting that host antibodies can target early gametocytes, and supporting the need for similar studies in other patient cohorts. Initial studies into gametocyte sequestration suggested that gametocytes participate in adhesive interactions with host cell receptors, and therefore that adhesins are likely expressed on the GIE surface. Adhesive properties of GIEs to C32 melanoma cells and trHBMEC human bone marrow endothelial cells were shown to only partly overlap with those of asexual parasites,

indicating gametocyte-specific sequestration mechanisms [44, 45]. Recent studies detected limited adhesion of gametocytes towards various endothelial cell types [46]. Building on recent evidence that gametocytes are enriched in the bone marrow parenchyma [9-11, 47], Joice *et al.* showed that the majority of bone marrow gametocytes in a cerebral malaria patient cohort localized specifically at erythroblastic islands, where young erythroblasts develop around a nursing macrophage [9]. Transcriptional data suggests that at least a subset of young gametocytes are in the blood circulation [8] while presence of asexual parasites in the bone marrow parenchyma and formation of gametocytes in erythroid precursor cells *in vitro* [9, 48] suggests that gametocyte formation could also occur in the bone marrow. In either case, potentially immunogenic surface molecules could be involved both in the processes of i) parasite binding to endothelial cells, and ii) erythroblast island binding in the bone marrow parenchyma. At the erythroblastic island, gametocyte proteins could be involved in binding either to erythroid precursor cells or macrophages. To further test these possibilities, binding experiments with different cell types could be performed and the ability of patient sera to inhibit these binding interactions examined. As cytokines and other innate immune factors likely influence endothelial permeability, it will be important to discern what role antibodies play compared to other immune components during both extravasation into and development within the bone marrow. Recent advances in distinguishing gametocyte stages *in vitro* [49, 50] and in flow cytometry assays examining antibody binding [51, 52] will enable identification of early stage gametocyte specific immune responses in diverse patient populations. The functionality of these immune responses could then be characterized in different clinical situations and the identity of the proteins targeted by early stage gametocyte-specific antibodies defined. Epitopes on erythrocytes infected with early stage gametocytes may form attractive novel targets for transmission blocking strategies. Functional recognition of early stage gametocytes could be exploited in vaccination strategies that interfere with homing to the bone marrow, maturation at this immunoprotective site and/or release of mature gametocytes into the blood stream

Late stage gametocyte infected erythrocyte surface antigens

In the first study of its kind, Saeed *et al.* observed naturally acquired humoral immunity against fully mature stage V gametocytes in serum samples of gametocyte-carrying Gambian children by flow cytometry [52]. This late GIE surface recognition increased with age and was most pronounced in serum samples taken at later time points during the infection when mature gametocytes appear in peripheral blood, but was unrelated to anti-asexual stage humoral immunity. Interestingly, immune responses to GIE were associated with decreased gametocyte density, suggesting that this type of humoral immunity may have consequences for gametocyte production or longevity. If confirmed in other studies, these findings hold great promise for the identification of novel mature gametocyte vaccine

targets. These confirmatory studies should take great care in preventing (low levels of) activation of these stage V gametocytes that would result in the accessibility of gamete antigens to which antibody responses may be highly prevalent in endemic settings [53-55].

The identification of gametocyte-specific epitopes on the GIE surface and their function remain to be established. Possible targets include multigene families located in the subtelomeric regions, which encode variant surface antigens known for their role in sequestration of asexual parasites and are also expressed in late stage gametocytes. STEVOR proteins have been identified as localized at the membrane of erythrocytes infected with late stage gametocytes [56] but a subset of the variant RIFIN antigens may also be targets of naturally acquired immunity against epitopes on the stage V GIE [57]. Another late stage gametocyte specific protein containing binding motifs is pSLAP [58]. Furthermore, the existence of signaling molecules expressed on the GIE and involved in spatial and temporal tropism (tropins and circadins) has been hypothesized [42].

Human immune responses active against mosquito-stage parasites

Since the majority of mature gametocytes die within the human host and fail to transmit to mosquitoes, proteins that are expressed by gametocytes but are only functional in activated gametes and later developmental stages are released and presented to the immune system. Indeed, many antigens present on the surface of these parasite stages (gametes, zygotes, ookinetes, oocysts) are already expressed in gametocytes in the human host, in preparation for gametogenesis [59]. Immune components that are taken up in the blood meal together with the gametocytes can inhibit fertilization in the mosquito midgut [60]. Once gametocytes leave the erythrocyte in the mosquito midgut, they are exposed to human cellular and humoral immune factors including leukocytes, antibodies and complement. In the avian malaria *P. gallinaceum*, transmission from chickens to mosquitoes was significantly reduced after vaccination of the birds with formalin-treated or irradiated gametes [61-63]. Also in the primate malaria *P. knowlesi*, evidence of induced TRI was observed upon immunization with gametocytes [64]. These animal models revealed for the first time that TRI could be induced, and was active against the parasite developmental stages occurring in the mosquito. Research into TRI and transmission blocking vaccine (TBV) development has since focused on immune responses to the mosquito stages of parasite development.

Mechanisms of mosquito-stage transmission blocking immunity

Leukocytes are capable of phagocytizing *Plasmodium* gametes, though the efficiency of this process appears lower in the mosquito midgut than during *in vitro* experiments, possibly because of lower temperature and enzymes present in the mosquito midgut [37, 38]. However, there is little evidence for the direct involvement of leukocytes in TRI, and conflicting evidence for a synergistic effect of cellular and antibody mediated immunity [65, 66].

Humoral immunity appears to be the dominant mechanism of natural TRI. The intra-host death of gametocytes that are not successfully transmitted releases a multitude of epitopes, including many present on the surface of the female macro- or male microgamete upon activation within the blood meal of a mosquito. Humoral immunity remains functional within the mosquito midgut and can inhibit transmission through a range of mechanisms. Specific antibodies may inhibit gamete fertilization either by agglutination resulting in inhibited gamete mobility, coating the micro- or macrogamete causing reduced cell-cell contact, opsonization for immune mediated lysis, or activation of the complement system resulting in gamete lysis [67-70]. Evidence for the dominance of antibody mediation in TRI development comes from its induction by vaccination with whole parasites [62-64] or from the activity of mAb specific to major sexual stage proteins [71]. The threshold at which humoral immunity significantly impacts transmission reduction, and the longevity of these responses are currently insufficiently explored.

Assessing immune responses that block mosquito infection

The best standardized method of determining the presence of TRI is the standard membrane feeding assay (SMFA) in which gametocytes are cultured *in vitro* and fed to laboratory-reared mosquitoes [72]. The direct membrane feeding assay (DMFA) is an alternative assay applicable in field situations, where gametocytes from naturally infected individuals are fed to mosquitoes either in the whole blood of the donor (to assess infectivity in the presence of other blood components), or after the removal and re-addition of autologous plasma or addition of plasma from a naive donor (to assess the impact of plasma components on transmission efficiency). These assays are described in detail elsewhere [72-74]. In both assays, the transmission reducing activity (TRA) of test sera/autologous plasma is generally determined one week after feeding when oocysts lining the mosquito midgut are visible by microscopy [75]. Evidence of functional TRI has generally been reported when the mean number of oocysts in test mosquitoes is decreased to less than 10% (TRA ≥90%) of that in control mosquitoes (mosquitoes fed the same gametocyte source with control serum [SMFA] or naïve plasma [DMFA]), while TRA <50% is considered evidence of limited or non-existent TRI [21, 76].

Advantages of the DMFA include the assessment of a multitude of locally relevant strains and more physiological gametocyte densities; however, large variation in these experiments does limit its predictive value and correlation with SMFA results. Transmission reduction in the DMFA was also correlated with gametocyte carriage, age above 5 years, and late season sampling, while it was inversely correlated with gametocyte density [77]. Several population-based screens using the DMFA in Cameroon, Kenya and the Gambia found that transmission efficiency is enhanced by 14-66% after replacement of autologous plasma with naive control serum [53-55, 77]. Although complete transmission blockage is rare, these findings suggest a relatively high prevalence of functional TRI in endemic

populations. However, non-specific factors (e.g. chemicals) present in the serum may contribute to the observed effect on TRA, and methodological issues including maintaining sample temperature during serum replacement may also affect transmission efficiency [78]. Non-specific factors can be excluded in the SMFA, as not only whole serum, but also specifically purified antibodies can be added to the infectious blood-meal. Naturally acquired transmission-reducing immunity has been detected using the SMFA with whole serum and purified IgG [53, 54, 79-81], although the proportion of individuals with reproducible TRI is generally much lower than observed in the DMFA with serum replacement. Variations in the intensity of mosquito infection arising from feeds on natural gametocyte carriers or cultured gametocytes make the assay outcomes difficult to compare, and may lead to a biological bias, as higher gametocyte density in the SMFA may increase epitope availability [82]. Efforts must therefore be made to standardise the conditions of the DMFA and SMFA. DMFA experiments require a better parameterization of inter-assay variability and procedural artifacts that may be interpreted as TRI. In turn, the SMFA can be optimized to better resemble natural conditions. The incorporation of multiple strains originating from various geographical locations could help avoid false-negative results due to sequence variation of gamete-specific epitopes in endemic strains while the use of gametocyte densities representative of natural mosquito infection rates would improve estimation of the impact of TRI on the likelihood of secondary infections [74]. Ideally, aiming to more accurately resemble the *in vivo* situation and avoid exhaustion of essential antibodies by non-specific binding to asexual and immature gametocytes currently present in the feeding material, only mature stage V gametocytes with sex ratios comparable to natural infections would be fed to mosquitoes.

A major drawback of both assays is their labor intensiveness, as the readout requires the dissection and midgut analysis of minimally 20 mosquitoes per condition after maintaining mosquitoes for a week. Recently, the use of a transgenic parasite line expressing luciferase enabled high throughput analysis of infection rates in mosquito midguts, which is a fundamental improvement for performing SMFA at a large scale [83].

Immune targets of transmission blocking immunity

Until the turn of the century, our understanding of protein expression during *P. falciparum* sexual development was limited to a selection produced in abundance at the onset of gametocytogensis (Pfg27/25 and Pfs16) [84, 85] or present on the gametocyte/gamete (Pfs230, Pfs48/45, Pfs41) [71] or zygote/ookinete (Pfs25, Pfs28) [86] surface. Many more proteins have now been identified as sexual stage specific [8, 29, 87, 88], with the best characterized described in previous reviews [59, 89, 90]. Although Pfs25 specific antibody responses have been observed in malaria exposed populations, Pfs25 and Pfs28 are post-transcriptionally repressed until the parasite's development in the mosquito midgut [59, 91], so it is unlikely that they would elicit functional TRI [92]. Naturally occurring

Table 1. Summary of studies correlating TRI assessed in the SMFA, with the presence of antibody responses specific to antigens Pfs48/45 and Pfs230

			Drakeley 2006 (Tanzania)		Bousema 2007 (Tanzania)		Bousema 2010b (Tanzania)		Drakeley 2004 (Gambia)		van der Kolk 2006 (Cameroon, Indonesia, Tanzania)		Bousema 2006 (PNG)		Combined	
Pfs48/45	TRA <50%	% Seropositive (n/N)	15.3	(9/59)	13.6	(6/44)	15.7	(8/51)	24.4	(10/41)	27.5	(33/120)	16.2	(19/117)	19.9	(85/432)
	TRA >90%		34.6	(9/26)	33.3	(2/6)	91.7	(11/12)	66.7	(2/3)	61.5	(8/13)	81.8	(9/11)	57.8	(41/71)
	OR (CI/p)		3.6	(1.16-11.12) 0.027	3.1	(0.6-17.2) 0.188	28.5	(3.3-246.7) 0.002	8.2	(0.6-113.9) 0.119	4.2	(1.3-13.8) 0.018	23.5	(3.8-146.3) 0.001	6.8	(3.7-12.4) <0.0001
Pfs230	TRA <50%	% Seropositive (n/N)	10.3	(6/58)	14.3	(6/42)	33.3	(17/51)	51.2	(22/43)	58.3	(91/156)	42.4	(50/118)	41.0	(192/468)
	TRA >90%		30.4	(7/23)	33.3	(2/6)	100	(12/12)	66.7	(2/3)	80	(20/25)	85.7	(6/7)	64.5	(49/76)
	OR (CI/p)		4.8	(1.3-17.5) 0.019	2.8	(0.4-18.0) 0.285	*nc*	(-)	2.6	(0.2-35.4) 0.463	3.0	(1.1-8.3) 0.040	10.0	(1.4-72.3) 0.022	4.8	(2.5-9.2) <0.0001
Pfs230 or Pfs48/45	TRA <50%	% Seropositive (n/N)	24.1	(14/58)	26.2	(11/42)	39.2	(21/51)	53.7	(22/41)	68.8	(75/109)	45.3	(48/106)	46.7	(190/407)
	TRA >90%		56.5	(13/23)	33.3	(2/6)	100	(12/12)	66.7	(2/3)	76.9	(10/13)	83.3	(5/6)	69.8	(44/63)
	OR (CI/p)		4.6	(1.6-13.3) 0.004	1.3	(0.3-7.0) 0.745	*nc*	(-)	2.2	(0.2-28.2) 0.547	1.5	(0.4-5.8) 0.571	6.4	(0.8-49.3) 0.073	3.8	(2.0-7.2) <0.0001

Table 1. Continued

Sample/population size	Total number of samples for which MFA and ELISA data for anti-Pfs230 or anti-Pfs48/45 responses were available/ Number of individuals from which samples were obtained. Three studies [76, 81, 102] were conducted longitudinally, giving multiple viable observations (n=1-4) for each individual. Exact sample sizes which vary between antigens.
TRA ≥90%	Sera reducing test mosquito mean oocyst intensity in the SMFA by ≥90% relative to oocyst intensity in control mosquitoes.
TRA <50%	Sera reducing test mosquito mean oocyst intensity in the SMFA by <50% relative to oocyst intensity in control mosquitoes.
% Seropositive (n/N)	Percentage of sera reducing transmission that are seropositive for antibodies specific to Pfs230 or Pfs48/45/(Number seropositive/total sample number)
OR (CI/p)	Odds ratio for functional TRI and seropositivity against Pfs48/45 or Pfs230 (Confidence intervals/p-value). TRI was considered evident if participant sera gave ≥90% TRA in the SMFA. Seropositivity in this 'transmission blocking' group was compared to seropositivity in individuals whose sera had <50% TRA. Sera reducing oocyst intensity by 50-90% were excluded from this analysis. For cross sectional studies [54, 80, 101] OR was calculated using standard logisitic regression. For studies with multiple observations per individual, study subject was incorporated into a generalized estimating equation (GEE) model as a random effect. For the combined analysis, study id was incorporated into a GEE model as a fixed effect and an exchangeable working correlation matrix was used for observations on the same subject. Robust standard errors were used. Adjustment for the age of sampled individuals did not affect effect estimates, nor was age an independent statistically significant predictor of TRI.
Pfs230 or Pfs48/45	To conduct analysis based on the recognition of either Pfs48/45 or Pfs230, only samples for which antibody responses to both antigens were assessed were included.
Combined	The results of all six studies for which data were available were combined to provide summary statistics for the association of anti-Pfs48/45 anti-Pfs230 responses and TRI.
nc	OR not calculable (100% of transmission blockers were reactive to the specified protein)

antibody mediated TRI is more likely the result of exposure to proteins expressed in the human parasite stages (so called pre-fertilisation antigens). Pfs230 and Pfs48/45 expression is initiated during gametocyte development and proceeds until fertilization of the micro- and macrogametes, with both proteins appearing to have an important role in male microgamete fertility [93, 94]. Antibodies specific to both proteins are naturally acquired after malaria infection [76, 95], and associated with varying levels of TRI; 14 studies have attempted to correlate TRI measured in MFA with immune recognition of Pfs230 and Pfs48/45, or sub-units thereof [53, 54, 76, 80, 81, 91, 96-103]. Of these 14 studies, nine determined the presence of antibody responses to Pfs230 and Pfs48/45 using enzyme-linked immunosorbent assay (ELISA), and related this data to serum TRA determined in the SMFA. Data was available for six studies, allowing a combined analysis of reactivity to these antigens in individuals with and without functional TRI (**Table 1**).

There is growing evidence from studies utilizing recombinant Pfs230 and Pfs48/45 that antibody responses to sexual stage antigens are acquired with age [80, 102, 103] in parallel with the development of blood-stage immunity [104]. Evidence for the age relatedness of TRI has been limited by age biased sampling. In the only study that assessed TRI for individuals of all ages, antigen-specific responses increased with age while the functionality of this response appeared to decrease [80]. More research is needed to improve our understanding of the acquisition and maturation of the TRI response in individuals of different ages across a range of transmission intensities.

Variability in the efficacy of sera to inhibit the transmission of different gametocyte isolates from naturally infected individuals suggests strain specific effects [99]. This might be due to antigenic variation, although Pfs48/45 and Pfs230 encoding genes show very limited variability in coding sequences [103]. Recently, immune sera from individuals in Mali were shown to recognize the male gamete protein HAP2, indicating expression by gametocytes in the human host. HAP2 specific mAb block transmission to mosquitos in *P. berghei* [105] and *P. falciparum* [92], but it has yet to be shown if the presence of anti-HAP2 Ab in endemic sera correlates with functional TRI.

While sera containing antibodies specific to Pfs48/45 or Pfs230 block transmission more commonly than sera with no effect on mosquito infection (Pfs48/45: OR 6.62 (3.61-12.15); Pfs230: OR 4.69 (2.50-8.81), recognition of these proteins does not predict blockage absolutely, nor does it confirm a mechanistic link with TRI. Several studies show no association between Ab response to Pfs230 and/or Pfs48/45 and transmission reduction [91, 97, 106, 107]. The presence of TRI in the absence of Pfs48/45 or Pfs230 specific antibodies, and a lack of TRI in their presence [53, 80, 97, 100-102] suggests that both may be partially functional, and that responses to multiple proteins could be necessary to elicit transmission inhibition. Combining the data in Table 1, 46.68% (190/407) of samples with <50% TRA recognized either Pfs48/45 or Pfs230, while 30.16% (19/63) of samples with TRA ≥90% recognized neither antigen. To definitively establish the role of naturally

occurring anti-Pfs48/45 and anti-Pfs230 antibodies in the development of TRI, specific antibodies should be affinity purified from immune sera using recombinant antigen and tested in the SMFA.

The association of sera transmission enhancement (TE) with anti-gamete immune responses has been a long standing discussion among researchers involved in TBV development. TE appears to occur naturally in a small proportion of gametocyte exposed individuals [91, 97, 99, 100, 108-110], though at lower frequency [101] (7% TE/48% TR) and relative intensity than naturally occuring transmission reduction. The cause of serum TE remains obscure: low antibody titres, from diluted immune sera or anti-gamete mAb have been linked with enhanced *P. vivax* transmission [108-110], and these findings are supported by the observation of enhancment during periods of low antibody carriage in the early and late stages of *P. cynomolgi* infection in Macaques [111]. Low titres of anti-gamete antibodies have also been linked with TE for *P. falciparum [97, 100, 112]*, however low titre responses to specific gamete and ookinete antigens (Pfs230, Pfs48/45, Pfs25, Pbs21) appear non-significantly [100] or not associated [101, 113, 114] with enhanced transmission, indicating that TE may be caused by a concurrent response to un-characterized gamete antigens, or by other serum factors. Investigating subtle reduction or enhancement of mosquito infection rate is made difficult by the SMFA's inherent variability, but in light of its potential impact on the efficacy of TBVs TE requires further investigation. Antibody responses to candidate TBVs provide an incomplete image of the immune signature of natural TRI, and if involved, these proteins probably represent only part of a larger range of immune responses contributing to TRI. The publishing of the *Plasmodium* genome in 2002 set the course for rapid advances in the fields of transcriptomics and proteomics. A wealth of studies employing high accuracy mass-spectrometry and mRNA microarrays identified proteins expressed specifically in gametocytes [115, 116], male and female gametocytes separately [88, 117], gametes, and ookinetes [118, 119]. The results of studies using *P. berghei* have been reviewed in detail [120]. Recently, high yield purification techniques allowed *P. falciparum* proteomic expression at the blood, early gametocyte, and late gametocyte stages to be disentangled, revealing the expression of >1400 proteins in early gametocytes, and >2000 in late gametocytes. Of these proteins, 1055 appear to be expressed in gametocytes but not in trophozoites, with 637 specific to stage IV and V gametocytes [87]. Proteomics approaches have confirmed the expression of known gametocyte surface proteins [121, 122], and identified hypothetical proteins likely to contain export sequences that may indicate surface expression [87]. Combined with analyses which have enabled the ranking of the *P. falciparum* proteome by the likelihood of possessing transmembrane domains or a glycophosphatidylinositol-anchor (GPI) [123], proteomics enables the prioritization of key candidates for involvement in antibody mediated TRI. With high throughput protein expression arrays the recognition by immune sera of thousands of proteins can now be assessed simultaneously [124, 125], and the results, as for previous studies with recombinant

Pfs230 and Pfs48/45, correlated to functional TRI measured in mosquito feeding assays. As the activity of many proteins (Pfs230 and Pfs48/45 included) is conformation dependent [126, 127], and as high throughput protein expression platforms are currently unable to produce tertiary protein structures, such an analysis is unlikely to provide a definitive list of antigens involved in functional TRI. However, it may still reveal new targets with less conformation-dependent activity, which would significantly further our understanding of TRI development and aid the rational design of a malaria TBVs.

Transmission blocking vaccines (TBV)

In recent years the development of the RTS,S malaria vaccine has received significant investment, with the results of a recent Phase 3 cluster randomized trial indicating that the vaccine may reduce the number of severe and un-complicated clinical cases in areas of high transmission [128]. Though highly effective pre-erythrocytic vaccines may be classified as vaccines that interrupt malaria transmission (VIMT) by preventing parasite multiplication and thereby gametocyte production, the limited longevity and efficacy of the response of RTS,S may be insufficient to significantly affect transmission in all endemic settings [128], and antibodies elicited by the vaccine have no direct impact on mosquito infection rate [129]. TBV development, reviewed by [89, 90, 130] has remained focused on six proteins against which antibodies (monoclonal, or from immunized sera) have been empirically shown to inhibit mosquito infection; *Plasmodium* proteins Pfs25 [86, 131], Pfs28 [86], Pfs230 [132], Pfs48/45 [126, 127] and more recently HAP2 [92, 105], and mosquito antigen AgAPN-1 [133]. If responses to pre-fertilisation antigens considered for TBV development are dominant effectors of naturally occurring TRI, vaccination efforts with these candidates may be aided by immune boosting from natural parasite exposure [134]. More research is required to improve our understanding of the temporal dynamics of sexual stage immunity, but limited evidence that TRI may be short-lived after gametocyte exposure [103] highlights the importance of prioritizing vaccine/adjuvant formulations that elicit long-lived immunity.

Whilst the advancement of Pfs25 to clinical trials is promising, examination of the WHO 'Rainbow tables' for malaria vaccine candidates currently in development makes it clear that candidate vaccines targeting transmission stage parasite remain vastly overshadowed by the number targeting alternative life cycle stages (pre-erythrocytic and asexual parasites). The value of a sexual stage, transmission blocking vaccine in the campaign to eliminate malaria is increasingly acknowledged, and the clinical testing of candidates other than Pfs25 and addition of novel targets are therefore urgently required. In addition to mosquito stage targets able to stimulate immune responses inhibiting parasite fertilization, increased focus should be on identifying targets integral to gametocyte development in the human host. Recent work providing insight into gametocyte development in the bone marrow parenchyma opens up new possibilities for immune responses targeting

immature gametocytes while field studies highlight the importance of responses targeting mature gametocytes in inducing TRI. Novel tools to prevent gametocyte maturation and longevity could contribute significantly to the interruption of malaria transmission. A better understanding of naturally occurring TRI and of the gametocyte's interaction with its human host is fundamental to the development of new TBV approaches.

Acknowledgments

WS and SR are supported by PATH Malaria Vaccine Initiative (MVI). WS and TB are supported by a Marie Curie Career Integration Grant from the European Community's Seventh Framework Programme (SIGNAL, PCIG12-GA-2012-333936).

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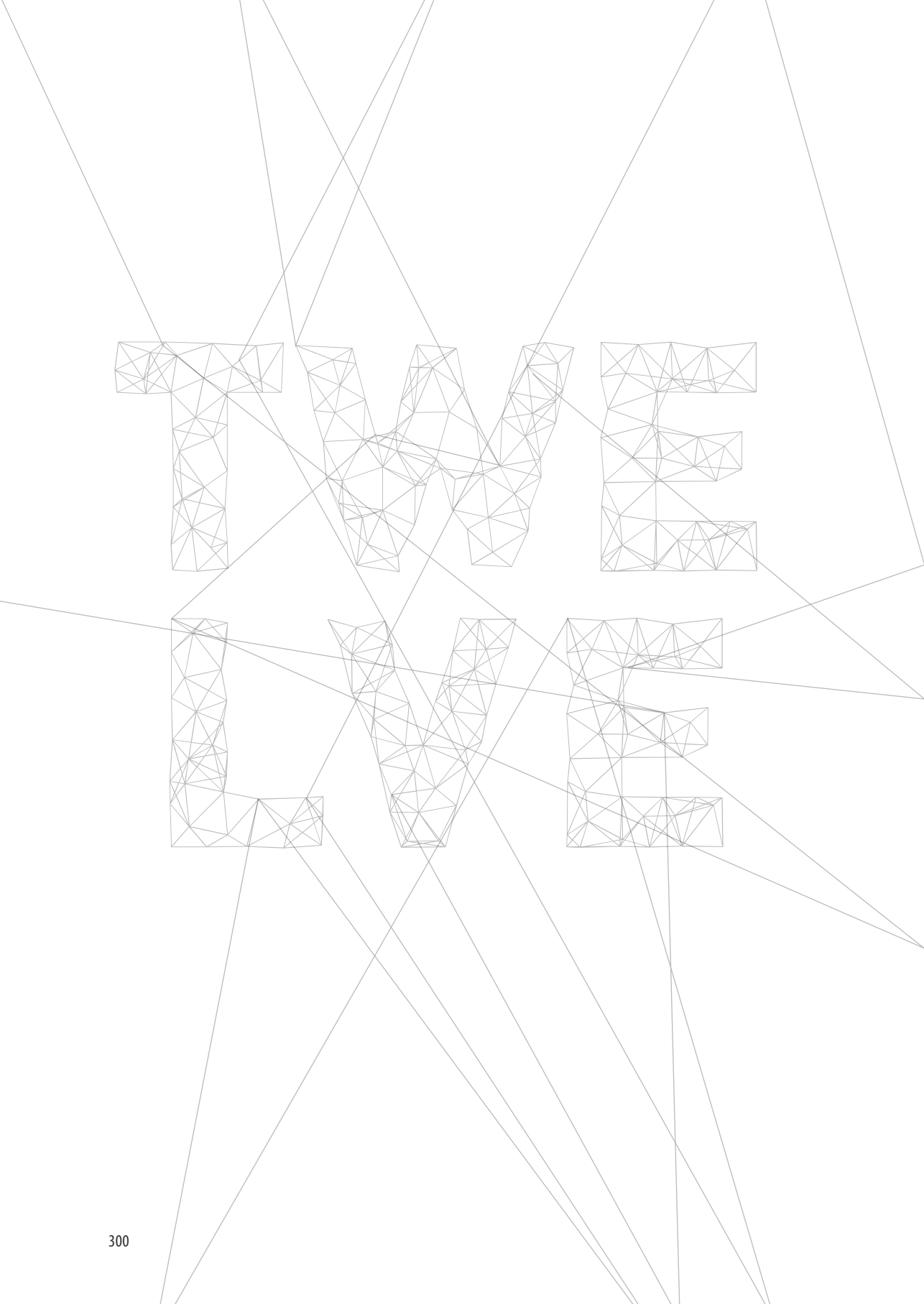
TWELVE

Chapter 12

Unravelling the immune signature of *P. falciparum* transmission blocking immunity

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Nature Communications (in press)

Abstract

Infection with *Plasmodium* can elicit antibodies that inhibit parasite survival in the mosquito, when they are ingested in an infectious blood meal. Here, we determine the transmission-reducing activity (TRA) of naturally acquired antibodies from 648 malaria-exposed individuals using lab based mosquito-feeding assays. Transmission inhibition is significantly associated with antibody responses to Pfs48/45, Pfs230, and to 43 novel gametocyte proteins assessed by protein microarray. In field-based mosquito-feeding assays the likelihood and rate of mosquito infection are significantly lower for individuals reactive to Pfs48/45, Pfs230, or to combinations of the novel TRA associated proteins. We also show that naturally acquired purified antibodies against key transmission-blocking epitopes of Pfs48/45 and Pfs230 are mechanistically involved in TRA, while sera depleted of these antibodies retain high-level, complement independent TRA. Our analysis demonstrates that host antibody responses to gametocyte proteins are associated with reduced malaria transmission efficiency from humans to mosquitoes.

Introduction

Plasmodium gametocytes develop from their asexual progenitors and are the only malaria parasite life-stage infective to mosquitoes. In preparation for their development in the mosquito, gametocytes cease to express many proteins involved in the parasites cycle of asexual replication, and upregulate others that are involved in sexual development [1, 2]. Some of these have essential roles in mosquito-stage development [3, 4], which culminates in the insect becoming infectious to humans.

In surveys where mosquitoes were fed directly on the skin or on a blood sample, it was noted that some gametocytaemic individuals including those with high gametocyte densities were not infectious [5, 6]. This may be associated with naturally acquired antibodies that interfere with parasite development within mosquitoes to reduce or prevent mosquito infection [7, 8]. The role of antibodies in determining transmission efficiency can be tested in mosquito feeding assays, in which purified antibodies are added to a mosquito blood meal that contains gametocytes [9, 10]. Evidence for naturally acquired transmission-reducing activity (TRA) is provided if test antibodies in endemic serum cause a reduction in the number of developing *Plasmodium* oocysts relative to mosquitoes fed the same infectious blood meal without test antibodies. TRA can be experimentally induced after immunisation of animals with inactivated *Plasmodium* gametocytes or gametes [8, 11]. The gametocyte and gamete proteins P48/45 and P230 have been identified as targets of transmission blocking monoclonal antibodies (mAb), which act by inhibiting the protein's role in gamete fertilisation in the mosquito gut [3, 4, 12]. Monoclonal antibodies specific to the zygote and ookinete proteins P25 and P28 were also shown to block transmission, by inhibiting ookinete invasion of the midgut epithelium [13]. All four have been produced as recombinant proteins that can induce antibody-mediated TRA in animal models [13-15].

Because P25 and P28 mRNA is translationally repressed until zygote formation [16], antibodies specific to these proteins appear absent in endemic populations [17, 18]. In contrast, responses to P48/45 and P230 are commonly observed in naturally infected individuals [10, 19, 20]. The presence of antibodies to *Plasmodium falciparum* Pfs48/45 and Pfs230 in endemic sera has been associated with TRA in several, but not all sero-epidemiological surveys [10, 21-28]. Importantly, many individuals with functional TRA do not have measurable antibodies against these two proteins [10, 23, 25, 28-31]. Despite these observations, there has been no attempt to demonstrate the contribution of naturally acquired α-Pfs48/45 and α-Pfs230 antibodies to transmission inhibition, and little investigation of alternative targets of natural TRA. The gametocyte proteome has now been described in detail [32, 33]. Utilising the protein microarray platform, genome scale datasets have been combined with functional and immunological data to provide valuable insight into mechanisms and markers of malaria humoral immunity [34, 35].

Here, we aim to investigate the immune signature of naturally occurring antibody-mediated TRA, to expand and prioritise antigenic targets for functional characterisation as biomarkers, or transmission-blocking vaccine candidates. To this purpose, we utilise a protein microarray comprising an inclusive selection of proteins expressed during gametocyte development. We assess antibody responses to these proteins and to correctly folded Pfs48/45 [36] and Pfs230 [15] in 648 malaria-exposed individuals from Burkina Faso, the Gambia, Cameroon, and from migrants from the Netherlands. Using purified total IgG, we assess the functional TRA of antibodies from the same individuals by determining their effect on mosquito infection density in standard membrane-feeding assays (SMFA) with cultured *P. falciparum* gametocytes and *Anopheles stephensi* mosquitoes. Our analysis reveals significant associations between high-level TRA and responses to Pfs48/45, Pfs230 and 43 novel gametocyte proteins. For a subset of 366 gametocyte positive donors who had provided blood samples to *Anopheles gambiae s.s.* or *Anopheles coluzzii* mosquitoes in field-based membrane-feeding assays, we determine the association of these antibody responses and mosquito infection rates during natural infections. For Pfs48/45 and Pfs230, we provide functional data that demonstrate their role in natural TRA. For one of the newly identified proteins (PfGEST) we provide experimental data that does not support its functional role in natural TRA.

Results

Antibody responses to gametocyte antigens

Plasma was collected in epidemiological studies in Burkina Faso [37-41], Cameroon [42, 43] and the Gambia [24, 44-47] [48], as well as from Dutch migrants who had lived for several years in malaria endemic areas and reported repeated malaria episodes (**Table 1**). Individuals from malaria endemic areas were all asymptomatic when sampled, and were either recruited randomly from the community (n=42), or based on the observation of malaria parasites (n=273) or specifically gametocytes (n=276) in blood smears. For a selection of individuals from malaria-endemic areas (n=498), infectivity to mosquitoes at the moment of sample collection was determined by offering a venous blood sample to *An. gambiae s.s.* or *An. coluzzii* in a direct membrane feeding assay (DMFA) (**Table 1**). Dutch migrants had no DMFA performed and had either recently returned from extended periods in endemic areas (within 6 months after return; n=8) or had returned more than a year before sampling (n=49). Those who had returned more than a year before sampling had lived in areas with endemic *P. falciparum* transmission for longer than 15 years, and reported between 2 and >80 clinical malaria episodes during that time.

Antibody responses to Pfs48/45 (Pfs48/45-10C [36]) and Pfs230 (230CMB[15]) were determined by enzyme-linked immunosorbent assay (ELISA) using recombinant, correctly

folded proteins. A protein microarray was constructed to assess responses to other gametocyte antigens. This array included a total of 315 proteins (**Supplementary Data 1**) that were broken up into 528 overlapping fragments <1000 amino acids in length (overlaps of 17 amino acids) for protein expression in an *Escherichia coli* based *in vitro* transcription-translation (IVTT) system [49, 50]. Antigens on the array included proteins previously implicated as eliciting TRA after immunisation, proteins involved in early gametocyte development as possible markers of gametocyte exposure [1], markers of asexual parasite exposure [51] and proteins expressed in gametocytes based on recent proteomic analysis [52]. To ensure that the array included most potential antibody targets involved in TRA, we preferentially included proteins that had transmembrane domains, signal peptides, or GPI anchors. Specificity of proteins to gametocytes was not pre-requisite for inclusion on the array, however 228/315 proteins had consensus evidence for enrichment in gametocytes based on a recent integration analysis of 11 available proteomics datasets [53] (**Supplementary Data 1**). Among donors from malaria endemic regions, the prevalence and magnitude of antibody responses to Pfs48/45 (Pfs48/45-10C [36]) and Pfs230 (230CMB[15]) increased with age (**Fig. 1 A-D**). In contrast, the overall breadth (linear regression [LinR]: $p<0.001$) and magnitude (analysis of variance [ANOVA]: $p<0.001$) of response to antigens on the microarray decreased with age (**Fig. 1 E-F**). The same associations were observed when analyses were restricted to donors from Burkina Faso (n=225), the site with the widest age range of donors (**Supplementary Fig. 1**).

Table 1. Sample characteristics

Sample origin (Study reference)	Samples (n)	Age (median, range)	Asexual positive (%)	Gametocyte positive (%)	Parasite free (%)	Infectious (DMFA, %)	TR activity <10%	≥50%	≥80%	≥90%
Gambia [24, 44-48]	226	5.0 (1.0-17.0)	99.1% (224/226)	74.2% (167/225)	0.4% (1/226)	24.0% (44/183)	54.0% (122/226)	15.9% (36/226)	4.4% (10/226)	3.1% (7/226)
Burkina Faso [24, 44-48]	225	16.0 (1.8-74.0)	56.5% (109/193)	53.5% (114/213)	31.9% (68/213)	45.5% (86/189)	68.4% (154/225)	8.0% (18/225)	2.7% (6/225)	1.8% (4/225)
Cameroon [42, 43]	140	8.5 (5.0-16.0)	90.5% (117/133)	93.5% (137/138)	0.0% (0/139)	73.8% (93/126)	68.6% (96/140)	14.3% (20/140)	6.4% (9/140)	4.3% (6/140)
The Netherlands [37-39, 43]	57	79.5 (26.0-84.0)	3.9% (2/51)	3.9% (2/51)	96.1% (49/51)	-	57.9% (33/57)	17.5% (10/57)	8.8% (5/57)	8.8% (5/57)
Combined	649	7.1 (1-92)	75.6% (458/606)	65.8% (413/628)	18.8% (118/629)	44.8% (223/498)	62.5% (405/648)	13.0% (84/648)	4.6% (30/648)	3.4% (22/649)

- data unavailable, or untested

TR activity % percent transmission reducing (TR) activity of purified IgG in the standard membrane-feeding assay (SMFA), relative to control mosquitoes fed the same gametocyte batch without test antibodies. TR activity is the mean of two independent SMFA runs for all samples with ≥80 % TR activity in the first run.

Samples total number of samples with TR activity assessed in the SMFA

Asex/Gct % asexual parasite and gametocyte prevalence by microscopy at the time of sampling, for individuals with available data

Parasite free % no asexual parasites or gametocytes observed by microscopy

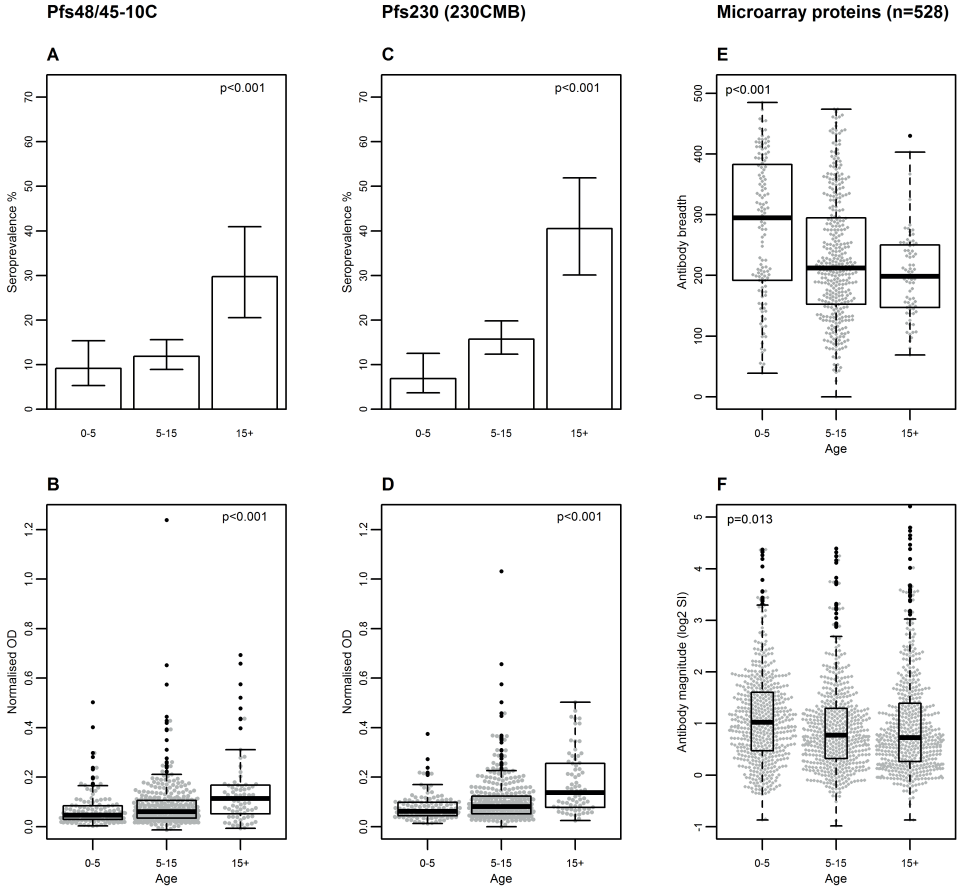


Figure 1. Antibody responses to Pfs48/45-10C, Pfs230 and to the microarray proteins with age. Antibody responses to correctly folded, recombinant Pfs48/45 and Pfs230 were measured using ELISA, with antibody intensity given as the ELISA optical density (OD) values (450nm). Antibody responses to microarray proteins are given as the $\log_2$-transformed signal intensity (SI) minus the vehicle SI, which equates to the $\log_2$-fold change over this background. All graphs show only individuals from endemic areas (Dutch migrants excluded). Sample size: 0-5 = 131, 5-15 = 366, 15+ = 71. **A & C:** Bars show the seroprevalence of α-Pfs48/45-10C and α-Pfs230 antibodies with age, with Clopper-Pearson confidence intervals. **B & D:** Box plots showing α-Pfs48/45-10C and α-Pfs230 antibody intensity among endemic individuals (Dutch migrants excluded) with age. **E**: Box-plots showing responses to the microarray protein targets (n=528). Antibody breadth is the number of proteins reactive above background in each individual, within the given groups. **F:** Magnitude of antibody response to microarray protein targets. Each dot represents the average SI of response to each protein target by all individuals within given groups. P-values for prevalence data are from likelihood ratio test for differences in seroprevalence between all age groups, derived from logistic regression and adjusted for gametocyte density. P-values for intensity data and response breadth are from an F test for differences in OD/SI between all age groups, derived from linear regression and adjusted for gametocyte density, or from ANOVA (for magnitude only). For all box-plots, outliers are shown in black while all data points are shown in grey as a bee-swarm.

Antibody responses associated with transmission-reducing activity (TRA)

To assess functional TRA, IgG was purified from the sera of all 648 individuals and provided to *An. stephensi* mosquitoes together with a blood meal containing cultured NF54 or transgenic NF54HT-GFP-Luc gametocytes in standard membrane-feeding assays (SMFA) [9, 54]. If the donor IgG caused ≥80% reduction in oocyst formation relative to control mosquitoes (TRA ≥80%), the SMFA was repeated with separate gametocyte culture material. In total, 3.3% (22/648) of the antibody samples caused replicable TRA of ≥90% (**Table 1**). On a continuous scale, TRA decreased with age, but was not significantly associated with gametocyte density at the point of sampling (LinR: p=0.269) (**Supplementary Fig. 2**). Because previous gametocyte exposure is associated with TRA [28], comparisons between sub-groups were nevertheless adjusted for gametocyte density. Though intermediate TRA may be relevant for transmission there are uncertainties about its reliable measurement in the SMFA [55, 56]. TRA was therefore dichotomized into ≥90% (high-level TRA) and <10% TRA (no TRA) before assessing associations with antibody responses. The group without evidence for TRA was refined by excluding individuals without microscopically detectable gametocyte densities at the time of sampling, resulting in 22 individuals with high-level TRA activity and 254 individuals who were exposed to gametocytes (and thus likely to respond to gametocyte antigens) but showed no evidence for TRA. Using these two categories, high-level TRA was significantly more likely in individuals seropositive for α-Pfs230 (odds-ratio [OR] from logistic regression [LogR] 7.9 [95% confidence interval [CI] 2.8-22.8], p<0.001), α-Pfs48/45 (OR 4.4 [95% CI 1.5-12.9], p=0.007), or either antigen (OR 5.90 [95% CI 2.1-16.7], p=0.001) (**Table 2; Supplementary Table 1**). Antibody density was also higher for Pfs230 (Lin R: p<0.001) and Pfs48/45 (p<0.001) in individuals exhibiting high-level TRA (**Fig. 2 A & B; Table 2**).

In addition to Pfs230 and Pfs48/45, the average magnitude of response to each microarray target was also higher in individuals with high-level TRA (students t-test: p=0.012) (**Fig. 2D**), whilst the breadth of response by each individual was not statistically significantly different between individuals with high-level TRA compared to those with no evidence of TRA (LinR: p=0.89) (**Fig. 2C**). Sixty microarray proteins had statistically significantly higher antibody magnitude in individuals with high-level TRA, after p-values had been adjusted to control the false discovery rate (FDR) (**Fig. 2E**) [57]. The inverse, higher responses in individuals with no TRA, was not observed (**Supplementary Data 2**). Logistic regression models with FDR controlled p-values showed that antibody prevalence to 27 microarray targets was significantly associated with high-level TRA, 25 of which were also significantly more reactive in the analysis of signal intensity. Because large proteins were fragmented on the array, the combined list of 62 novel microarray targets with TRA associated antibody responses in either the linear (response intensity) or logistic (response prevalence) analysis represented fragments of 43 unique proteins (**Table 2**). 15 of

the 43 proteins with TRA associated responses are conserved unknown proteins. Two are known gametocyte/gamete proteins with putative roles in gamete egress; PF3D7_1038400 (gametocyte specific protein, Pf11-1) and PF3D7_1449000 (gamete surface and sporozoite traversal protein/GEST).

TRA-associated responses on the array showed only partial overlap with Pfs48/45 and Pfs230 responses. Individuals with high-level TRA who were seronegative for both α-Pfs230 and α-Pfs48/45 (n=10/22), had antibody responses to significantly more TRA associated microarray targets (median breadth 11 (out of 62) [interquartile range [IQR] 3-26]) than gametocyte positive individuals without TRA (n=211; median breadth 4 (out of 62) [IQR 2-9), Lin R: p=0.018). Responses to some TRA biomarkers were highly prevalent in individuals with high-level TRA who lacked significant responses to Pfs45/45 and Pfs230 (e.g. Pf11-1 [PF3D7_103840] = 70% [n/N=7/10]; LSA-3 [PF3D7_0220000] = 80% [n/N=8/10]). Responses to one target protein mapping to PF3D7_1103800 (CCR4-NOT) were present in 60% (n/N=6/10) of high level TRA α-Pfs48/45 and α-Pfs230 seronegatives, while seroprevalence for this target in individuals with no TRA was 10.3% (n/N=26/254) (**Supplementary Data 3**).

Interestingly, 10 of the 43 proteins with TRA associated antibody responses are putatively exported during early gametogenesis [1]. Though the majority of these proteins are enriched in asexual parasites, their association with TRA may reflect previous observations that TR immunity is induced and wanes rapidly after gametocyte exposure [26, 27]. Responses to putative markers of asexual parasite exposure (PF3D7_1036000 [Merozoite surface protein 11 [MSP11]), PF3D7_0711700 (erythrocyte membrane protein 1, PfEMP1 [VAR]), PF3D7_0731600 (acyl-CoA synthetase [ACS5]), and PF3D7_0423700 (early-transcribed membrane protein 4 [ETRAMP4]) [51] were not associated with TRA, either in terms of response intensity (Bayes empirical t-test: FDR adjusted p=0.34-0.99) or seroprevalence (LogR: FDR adjusted p=1).

Table 2. TRA associated antibody responses assessed by ELISA or protein microarray

Gene ID	Gene description	Seropositivity				Signal intensity	
		Seropositivity % (n/N) [TRA ≥90%)	Seropositivity % (n/N) [TRA <10%)	OR	p-value	Fold change SI	p-value
PF3D7_0209000*	6-cysteine protein (P230)	50% (11/22)	10.6% (27/254)	7.9	<0.001	2.5	<0.001
PF3D7_1346700*	6-cysteine protein (P48/45)	40.9% (9/22)	11.4% (29/254)	4.4	0.007	2.1	<0.001
PF3D7_0826100.a	E3 ubiquitin-protein ligase, putative	59.1% (13/22)	11.8% (30/254)	10.8	<0.001	2.5	<0.001
PF3D7_1103800.a	CCR4-NOT transcription complex subunit 1, putative (NOT1)	36.4% (8/22)	3.5% (9/254)	15.6	<0.001	1.6	<0.001
PF3D7_0936400	ring-exported protein 4 (REX4)	59.1% (13/22)	15% (38/254)	8.2	0.001	2.2	<0.001
PF3D7_0102200.a	ring-infected erythrocyte surface antigen (RESA)	50% (11/22)	11% (28/254)	8.1	0.001	2.6	<0.001
PF3D7_1103800.b	CCR4-NOT transcription complex subunit 1, putative (NOT1)	45.5% (10/22)	10.2% (26/254)	7.3	0.003	1.5	<0.001
PF3D7_1324600	conserved Plasmodium protein, unknown function	54.5% (12/22)	16.1% (41/254)	6.2	0.004	1.8	<0.001
PF3D7_1149200.a	ring-infected erythrocyte surface antigen	45.5% (10/22)	13% (33/254)	5.6	0.012	2.5	<0.001
PF3D7_0322600	conserved Plasmodium protein, unknown function	22.7% (5/22)	3.1% (8/254)	9.0	0.019	1.3	<0.001
PF3D7_0826100.b	E3 ubiquitin-protein ligase, putative	18.2% (4/22)	1.6% (4/254)	13.9	0.019	1.4	<0.001
PF3D7_0930500.a	diacylglycerol kinase, putative (DGK1)	45.5% (10/22)	15% (38/254)	4.7	0.026	1.7	<0.001
PF3D7_1103800.c	CCR4-NOT transcription complex subunit 1, putative (NOT1)	36.4% (8/22)	9.8% (25/254)	5.2	0.026	1.5	<0.001
PF3D7_1010700	dolichyl-phosphate-mannose protein mannosyltransferase, putative	22.7% (5/22)	4.3% (11/254)	6.5	0.044	1.3	<0.001
PF3D7_1038400.a	gametocyte-specific protein (Pf11-1)	31.8% (7/22)	8.7% (22/254)	4.9	0.044	1.6	<0.001
PF3D7_0505000	conserved Plasmodium membrane protein, unknown function	22.7% (5/22)	7.1% (18/254)	3.9	0.156	1.6	<0.001
PF3D7_1433200.a	conserved Plasmodium protein, unknown function	22.7% (5/22)	8.3% (21/254)	3.3	0.246	1.4	<0.001
PF3D7_1360500	guanylyl cyclase beta (GCbeta)	40.9% (9/22)	15% (38/254)	3.9	0.062	1.5	0.001
PF3D7_0102200.b	ring-infected erythrocyte surface antigen (RESA)	59.1% (13/22)	29.5% (75/254)	3.4	0.093	2.4	0.002
PF3D7_0301700	Plasmodium exported protein, unknown function	59.1% (13/22)	18.1% (46/254)	6.5	0.004	2.0	0.004
PF3D7_1212100	peripheral plastid protein 1, putative (PPP1)	40.9% (9/22)	16.9% (43/254)	3.4	0.102	1.7	0.004
PF3D7_0731800	alpha/beta hydrolase, putative (GEXP08)	40.9% (9/22)	7.5% (19/254)	8.6	0.002	1.6	0.005

Table 2. Continued

PF3D7_1038400.b	gametocyte-specific protein (Pf11-1)	63.6% (14/22)	26.8% (68/254)	4.8	0.026	2.0	0.005
PF3D7_0220000	liver stage antigen 3 (LSA3)	81.8% (18/22)	44.5% (113/254)	5.6	0.049	1.9	0.005
PF3D7_0804500.a	conserved Plasmodium membrane protein, unknown function	22.7% (5/22)	4.7% (12/254)	5.9	0.049	1.4	0.005
PF3D7_1107900	mechanosensitive ion channel protein	40.9% (9/22)	12.6% (32/254)	4.8	0.028	1.4	0.006
PF3D7_0726400.a	conserved Plasmodium membrane protein, unknown function	45.5% (10/22)	19.7% (50/254)	3.4	0.094	1.5	0.006
PF3D7_0930500.b	diacylglycerol kinase, putative (DGK1)	31.8% (7/22)	7.9% (20/254)	5.5	0.028	1.4	0.009
PF3D7_0628200	protein kinase PK4 (PK4)	45.5% (10/22)	16.9% (43/254)	4.1	0.049	1.5	0.009
PF3D7_0826100.c	E3 ubiquitin-protein ligase, putative	45.5% (10/22)	21.7% (55/254)	3.0	0.148	1.7	0.010
PF3D7_1127500	protein disulfide isomerase, putative	36.4% (8/22)	16.1% (41/254)	3.0	0.184	1.4	0.010
PF3D7_1314500	cop-coated vesicle membrane protein p24 precursor, putative	45.5% (10/22)	23.6% (60/254)	2.7	0.219	1.8	0.010
PF3D7_1038400.c	gametocyte-specific protein (Pf11-1)	86.4% (19/22)	48% (122/254)	6.9	0.049	2.2	0.011
PF3D7_0922100	ubiquitin-like protein, putative	9.1% (2/22)	0.8% (2/254)	12.6	0.137	1.2	0.012
PF3D7_0702300	sporozoite threonine and asparagine-rich protein (STARP)	63.6% (14/22)	42.5% (108/254)	2.4	0.351	1.7	0.012
PF3D7_0523400	DnaJ protein, putative	31.8% (7/22)	10.6% (27/254)	3.9	0.093	1.7	0.013
PF3D7_0305300	conserved Plasmodium membrane protein, unknown function	36.4% (8/22)	17.3% (44/254)	2.7	0.246	1.7	0.013
PF3D7_0603600	conserved Plasmodium protein, unknown function	4.5% (1/22)	0% (0/254)	*nc*	*nc*	1.2	0.013
PF3D7_0306400	FAD-dependent glycerol-3-phosphate dehydrogenase, putative	40.9% (9/22)	17.7% (45/254)	3.2	0.124	1.5	0.014
PF3D7_1433200.b	conserved Plasmodium protein, unknown function	45.5% (10/22)	20.9% (53/254)	3.2	0.124	1.5	0.014
PF3D7_0726400.b	conserved Plasmodium membrane protein, unknown function	22.7% (5/22)	3.5% (9/254)	8.0	0.026	1.2	0.015
PF3D7_1326500	conserved Plasmodium protein, unknown function	18.2% (4/22)	7.9% (20/254)	2.6	0.524	1.4	0.015
PF3D7_0804500.b	conserved Plasmodium membrane protein, unknown function	45.5% (10/22)	18.9% (48/254)	3.6	0.085	1.5	0.016
PF3D7_0830600	Plasmodium exported protein (PHISTc), unknown function	40.9% (9/22)	17.7% (45/254)	3.2	0.124	1.7	0.016
PF3D7_0801000	Plasmodium exported protein (PHISTc), unknown function	4.5% (1/22)	0.4% (1/254)	12.0	0.422	1.7	0.016

Table 2. Continued

PF3D7_1021100	conserved Plasmodium protein, unknown function	27.3% (6/22)	16.5% (42/254)	1.9	0.799	1.5	0.017
PF3D7_1024800	conserved Plasmodium protein, unknown function	40.9% (9/22)	16.5% (42/254)	3.5	0.094	1.5	0.020
PF3D7_0826100.d	E3 ubiquitin-protein ligase, putative	36.4% (8/22)	18.9% (48/254)	2.5	0.344	1.5	0.021
PF3D7_1149200.b	ring-infected erythrocyte surface antigen	36.4% (8/22)	22.4% (57/254)	2.0	0.642	1.7	0.024
PF3D7_1306500	MORN repeat protein, putative	31.8% (7/22)	10.2% (26/254)	4.1	0.085	1.3	0.025
PF3D7_0815300	FAD-dependent monooxygenase, putative	45.5% (10/22)	26.4% (67/254)	2.3	0.351	1.6	0.026
PF3D7_0726400.c	conserved Plasmodium membrane protein, unknown function	40.9% (9/22)	23.6% (60/254)	2.2	0.422	1.5	0.026
PF3D7_1104700.2	RNA polymerase subunit, putative	13.6% (3/22)	0.8% (2/254)	19.9	0.042	1.1	0.028
PF3D7_1143700	conserved Plasmodium protein, unknown function	27.3% (6/22)	8.3% (21/254)	4.2	0.094	1.2	0.032
PF3D7_0826100.e	E3 ubiquitin-protein ligase, putative	36.4% (8/22)	17.7% (45/254)	2.7	0.274	1.5	0.032
PF3D7_0826100.f	E3 ubiquitin-protein ligase, putative	40.9% (9/22)	22.4% (57/254)	2.4	0.344	1.3	0.032
PF3D7_1449000	gamete egress and sporozoite traversal protein, putative (GEST)	22.7% (5/22)	11% (28/254)	2.4	0.532	1.3	0.035
PF3D7_0726400.d	conserved Plasmodium membrane protein, unknown function	31.8% (7/22)	13% (33/254)	3.1	0.183	1.5	0.036
PF3D7_0323100	conserved Plasmodium protein, unknown function	36.4% (8/22)	16.9% (43/254)	2.8	0.223	1.5	0.036
PF3D7_0804500.c	conserved Plasmodium membrane protein, unknown function	31.8% (7/22)	5.1% (13/254)	8.7	0.004	1.4	0.043
PF3D7_0826100.g	E3 ubiquitin-protein ligase, putative	27.3% (6/22)	11.4% (29/254)	2.9	0.274	1.4	0.044
PF3D7_1354200	inositol-polyphosphate 5-phosphatase, putative (IP5P)	40.9% (9/22)	18.9% (48/254)	3.0	0.168	1.5	0.045
PF3D7_1014300	conserved protein, unknown function	27.3% (6/22)	6.7% (17/254)	5.2	0.049	1.2	0.117
PF3D7_1348000	conserved Plasmodium protein, unknown function	13.6% (3/22)	1.2% (3/254)	13.2	0.049	1.1	0.310

Table 2. Continued

Gene ID	Proteins, ordered by effect size and significance in the bayes-moderated t-test/linear regression analysis, and then by significance in logistic regression. Letters indicate cases where multiple protein targets (fragments) mapping to the same proteins are associated independently with TRA.
Fold change SI	Absolute fold change in average signal intensity (SI) of response to individual proteins by individuals with <10% TRA (gametocyte positive) and ≥90% TRA (1=no change)
Seropositivity	Seropositivity was calculated using protein specific cut-offs, calculated using maximum likelihoods methods
p-values	P-values after Benjamini-Hochberg adjustment to control the rate of false discovery below 5%, except for Pfs230 and Pfs48/45 which were assessed independently.
OR	Odds-ratio
TR	Transmission reduction
*	Pfs48/45 and Pfs230 data are from ELISA, with correctly folded proteins. Signal intensity comparisons were made by linear regression, seropositivity analysis by logistic regression. Fold change SI is the fold change of the mean normalised OD between individuals with TRA <10% and >90%. Because these serological assays were independent, p values are not controlled for false discovery

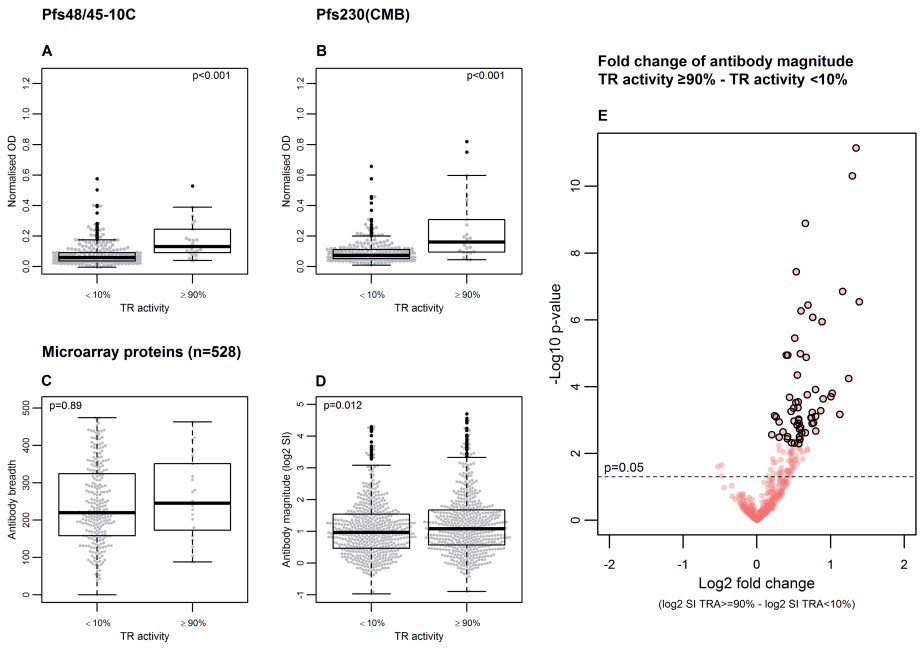


Figure 2. Antibody responses to Pfs48/45-10C, Pfs230 and to the microarray proteins with transmission reducing activity (TRA). TRA was categorised as described, to compare responses between gametocyte positive individuals with <10% TRA, and individuals with >90% TRA. Antibody responses to correctly folded, recombinant Pfs48/45 and Pfs230 were measured using ELISA, with antibody intensity given as the ELISA optical density (OD) values (450nm). Antibody responses to microarray proteins are given as the log_2-transformed signal intensity (SI) minus the vehicle SI, which equates to the log_2-fold change over this background. **A & B:** Box-plots of α-Pfs48/45-10C and α-Pfs230 antibody intensity with TRA. **C**: Responses to the microarray protein targets (n=528). Antibody breadth is the number of proteins reactive above background in each individual, within the given groups. **D:** Magnitude of antibody response to microarray protein targets. Each dot represents the average SI of response to each protein target by all individuals within given groups. P values for intensity data and response breadth are from an F test for differences in OD/SI between all age groups, derived from linear regression, and adjusted for gametocyte density, or from students t-test (for magnitude only). For all box-plots, outliers are shown in black while all data points are shown in grey as a bee-swarm. **E:** Volcano plot showing the log_2-fold change of the mean signal intensity for TRA as defined above. The p-value shown by the dotted line is unadjusted for false discovery. Black circled data points are those that remain significant after p-values have been adjusted to control the rate of false discovery below 5%, using the Benjamini-Hochberg method.

TRA and mosquito infection rate in natural infections

Next, we determined whether the immune profile associated with TRA in the SMFA was also associated with mosquito infection rates observed in direct-membrane feeding experiments. Data on the infectivity of donor blood samples to mosquitoes at the time of plasma donation was available for 494 individuals for whom gametocyte status had been assessed by microscopy and TRA assessed in the SMFA. Of the 128 individuals without patent gametocytaemia, 18 (14.1%) were infective to mosquitoes in the DMFA. Of the 366 gametocyte positive individuals, 203 (55.5%) infected at least one mosquito, with a median mosquito infection rate of 20% (IQR 7.7-42.5%). There was good agreement between transmission outcomes in the SMFA using cultured gametocytes and *An. stephensi* mosquitoes, and the field-based DMFA using gametocytes in infected individuals and *An. gambiae s.s.* or *An. coluzzii* mosquitoes; Individuals with high-level TRA in the SMFA were significantly less likely to infect any mosquitoes in the DMFA (LogR: OR 0.23 [95% CI 0.06-0.79], p=0.020), and more likely to do so at a reduced rate (OR 0.10 [95% CI 0.2-0.61], p=0.012). The likelihood of gametocyte positive individuals infecting any mosquitoes in the DMFA was significantly lower if seropositive for Pfs230 (LogR: OR 0.42 [95% CI 0.22-0.78], p=0.006), Pfs48/45-10C (OR 0.30 [95% CI 0.16-0.59], p<0.001), or either or both antigens (OR 0.33 [95% CI 0.19-0.58], p<0.001) (**Fig. 3A**). The proportion of mosquitoes that became infected after feeding was also significantly lower for individuals seropositive for Pfs230 (LogR: OR 0.36 [95% CI 0.14-0.88], p<0.027), Pfs48/45-10C (OR 0.14 [95% CI 0.05-0.40], p<0.001) or either or both antigens (OR 0.25 [95% CI 0.11-0.55], p=0.001) (**Fig. 3B**). The proportion of mosquitoes that became infected decreased with increasing Pfs230 antibody density (LogR: OR for an increase of 0.1 normalised optical density 0.59 [95% CI 0.40-0.86], p=0.007) and Pfs48/45-10C antibody density (OR 0.43 [95% CI 0.28-0.66], p<0.001).

Mosquito infection prevalence was lower in gametocyte positive individuals with antibodies recognising more of the 62 novel protein microarray targets with TRA associated antibody responses (**Supplementary Fig. 3**). Individuals responding to ≥14/62 microarray proteins (14 being the 75th percentile of breadth of response to these targets) were significantly less likely to infect mosquitoes (LogR: OR 0.31 [95% CI 0.18-0.51], p<0.001), and more likely to do so at a reduced rate (OR 0.21 [95%CI 0.09-0.45]. p<0.001) (**Fig. 3 A & B**). After excluding Pfs48/45 or Pfs230 seropositive individuals, infectiousness and infection rate remained significantly lower in individuals responding to ≥14 of the 62 TRA associated microarray proteins (infectiousness: LogR, OR 0.29 [95% CI 0.16-0.53], p<0.001, infection rate: OR 0.23 [95% CI 0.10-0.52], p<0.001).

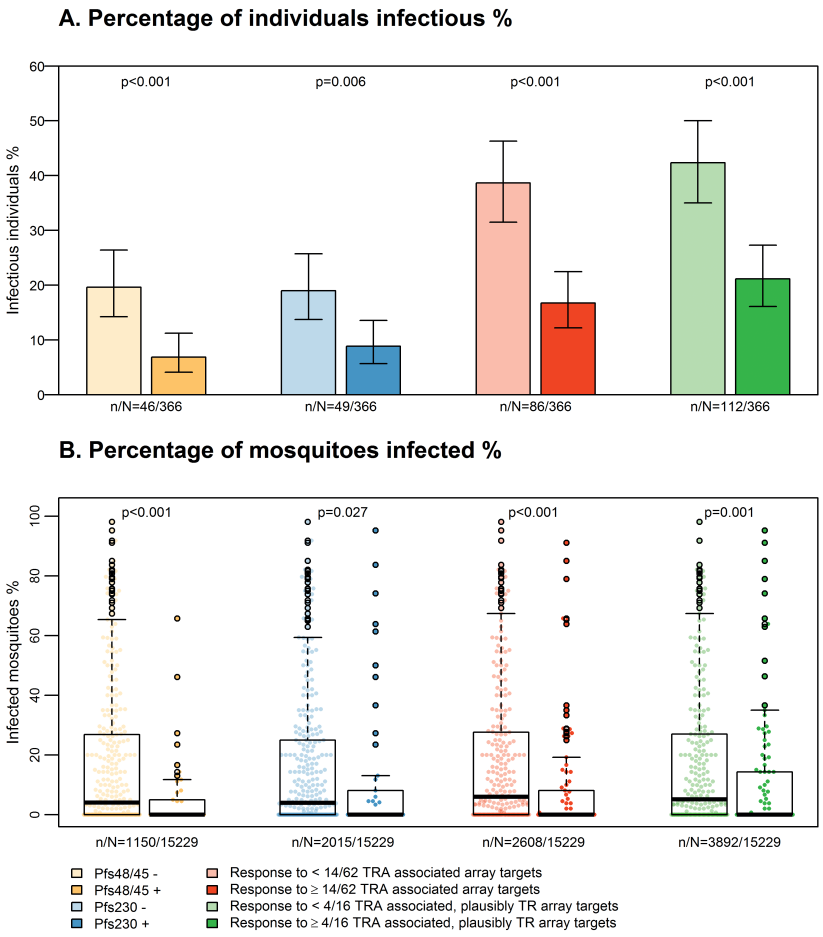


Figure 3. Seroprevalence to Pfs48/45, Pfs230, and novel TRA associated microarray proteins, and infectiousness in the direct membrane-feeding assay (DMFA). Individuals with DMFA data were categorised according to their possession of antibodies specific to: Pfs48/45 (positive [+]/negative [-]); Pfs230 (positive [+]/negative [-]); ≥14 of the 61 novel microarray proteins with TRA associated antibody responses (15 being the 75th percentile of the breadth of response to these microarray targets among the entire sample set); ≥4 of the 16 novel microarray proteins with TRA associated antibody responses that are also plausible targets of antibodies with TRA (4 being the 75th percentile of the breadth of response to these microarray targets among the entire sample set). **A:** Bars show the proportion of infectious individuals among seropositive/seronegative gametocytaemic individuals with DMFA data, with Clopper-Pearson confidence intervals. n/N = number of individuals seropositive/total number of individuals with DMFA data. P-values are from logistic regression, with adjustment for gametocyte density. **B:** Box-plots show the percentage of mosquitoes that became infected after feeding. n/N = the number of mosquitoes feeding on seropositive individuals/the total number of mosquitoes feeding on individuals with DMFA data. P-values are from logistic models, adjusted for gametocyte density and with host (individual the mosquitoes were feeding upon) as a random effect. For all box-plots, outliers are shown as hollow black circles while all data points are shown as solid coloured dots.

Functional involvement of antibodies in TRA

To demonstrate whether naturally-acquired antibodies to Pfs230 or Pfs48/45 were causally associated with TRA, we affinity-purified antibodies specific to key transmission-blocking epitopes of these proteins and assessed their activity in the SMFA (**Table 3**). These experiments were performed for 6 donors whose IgG showed high-level TRA and for whom high volumes of plasma (≥1mL) were available; 3 of these donors had ≥10mL plasma available, allowing additional testing after 9-fold antibody concentration of IgG. The flow-through of affinity purification experiments, depleted of α-Pfs230 and α-Pfs48/45 antibodies, was also tested in the SMFA to quantify TRA of antibodies to other target antigens. Concentrated back to the original plasma volume, α-Pfs48/45 antibodies of one donor independently inhibited transmission (TRA 91.5% [95% CI 86.4-94.7]), while α-Pfs230 antibodies of a different donor had low but statistically significant TRA (TRA 41.1% [95% CI 15.5-60.4]). When antibodies were concentrated to nine times their physiological concentration (performed for 3 donors with >9mL plasma available), α-Pfs48/45 antibodies from one additional individual, and α-Pfs230 antibodies from two additional individuals significantly reduced transmission to mosquitoes (Pfs48/45: TRA 81.3%, [95% CI 70.9-88.0], Pfs230: TRA 94.8% [95% CI 90.2-97.3] & 99.3% [95% 98.6-99.6], **Table 3**). For all individuals the α-Pfs48/45 and α-Pfs230 depleted IgG (containing no antibodies against Pfs48-45 epitopes 1-3, or Pfs230 epitope C, confirmed by ELISA) inhibited transmission to mosquitoes (**Table 3**). This activity was complement-independent.

To determine the presence of naturally-acquired antibodies binding to unknown gamete surface antigens, we performed surface immuno-fluorescence assays (SIFA) using gametes of a Pfs48/45 knock-out (KO) parasite [3] that does not produce surface retained Pfs230 [12]. Pfs48/45 and Pfs230 specific antibodies confirmed the absence of these proteins on Pfs48/45 KO gamete surfaces (**Fig. 4**). The immuno-fluorescence of gamete surfaces observed using total IgG and IgG depleted of α-Pfs48/45 and α-Pfs230 antibodies from naturally exposed individuals therefore reflects recognition of unknown antigens on the gamete surface (**Fig. 4, Supplementary Fig. 5A**).

Table 3. Activity of affinity purified antibodies against R0-10C (Pfs48/45) and 230CMB (Pfs230) from transmission reducing sera

ID	Location	Sex/Age	Time since malaria exposure	TRA% (95% confidence intervals)							
				Pfs48/45-10C (R0-10C)				2. Pfs230 (230CMB)			
				1. Total IgG	2. Pure IgG	*Conc. IgG*	3. Total IgG (- R0-10c Ab)	4. Pure IgG	*Conc. IgG*	5. Total IgG (- R0-10C & 230CMB IgG)	5. Total IgG (- R0-10C & 230CMB IgG) (C-)
A	Netherlands	M/69	Infected	99.4 (98.9-99.7)	91.5 (86.4-94.7)		99.5 (99-99.7)	-2.5* (-38.6-24.2)	99.3 (98.6-99.6)	99.5 (99-99.8)	99.8 (99.5-99.9)
B	Netherlands	-	Infected	99.6 (99.3-99.8)	-42.8 (-80.5--12.9)		96.7 (95.6-97.5)	-15.7* (-46.1-8.4)		88.5 (85.7-90.7)	87 (79.9-91.6)
C	Netherlands	-	<6 months	99.9 (99.7-100)	25.6* (-4.1-46.8)	81.3 (70.9-88)	99.4 (99-99.7)	42.1 (15.5-60.4)	94.8 (90.2-97.3)	89.1 (80.2-94)	95.8 (91.4-98)
D	Netherlands	M/74	~5 years	99.6 (99-99.8)	-14.7* (-65.3-20.5)	-0.3* (-38.7-27.4)	85.4 (75.4-91.3)	32* (-1-54.1)	0.1* (-58.8-37.1)	71.8 (52.7-83.2)	80.8 (66.8-88.9)
E	Cameroon	F/12	Infected	98.8 (98-99.3)	41.7 (26-54.1)		93.1 (90.3-95.1)	16.5* (-8.6-35.8)		92.8 (89-95.3)	93.4 (88.6-96.1)
F	Cameroon	F/6	Infected	100 (99.8-100)	29.3* (-6.7-53.2)		99.9 (99.7-100)	14.8* (-22.1-40.6)		97.2 (95.5-98.3)	98.9 (96.7-99.6)
Ctrl	Netherlands	Pooled	Never	-26.5* (-72-7)	-17.2* (-50.9-9)	4.6* (-33-31.5)	-14.7* (-50-12.3)	-6.3* (-31.4-14)	-2.7* (-41.5-25.5)	6.5* (-19.4-26.9)	

Transmission reducing activity is from duplicate SMFA experiments, with luminescence intensity 7-9 days after mosquito feeding (using NF54HT-GFP-luc strain gametocytes) as the output measure of infection intensity. All samples were tested in the presence of complement unless noted otherwise (C-). Total IgG represents the SMFA whole purified patient IgG at physiological concentration. Pure Ab represents the TRA of antibodies that bound to the R0-10c and 230CMB columns. Conc. Ab represents pure antibodies at 9 times the physiological concentration. The fractions tested were as follows; 1. Total IgG, 2. Purified α-R0-10c IgG, 3. α-R0-10c depleted IgG, 4. α-230CMB IgG, 5. α-R0-10c and α-230CMB depleted total IgG. Fraction 5, depleted of antibodies binding to either protein column, was tested with and without (C-) complement. Ctrl is IgG from plasma from pooled naive Dutch donors. All TR values and confidence intervals were calculated from two independent SMFAs with different culture material. TRA, CI, and p-values were calculated using generalised linear models (GLM) as described previously [72]. Asterisks (*) indicate results in which the oocyst/luminescence intensity of the test mosquitoes was not significantly different to the controls. Corrected ELISA OD values for α-10c Ab in: 1. A = 0.12, B = 0.88, C = 0.15, D = 0.08, E = 0.13, F = 0.08, Ctrl = 0.02; 3. 0.01-0.04. Corrected ELISA OD values for α-230CMB Ab in: 1. A = 0.05, B = 1.16, C = 0.76, D = 0.27, E = 0.10, F = 0.07, Ctrl = 0.06; 5. 0.01-0.03.

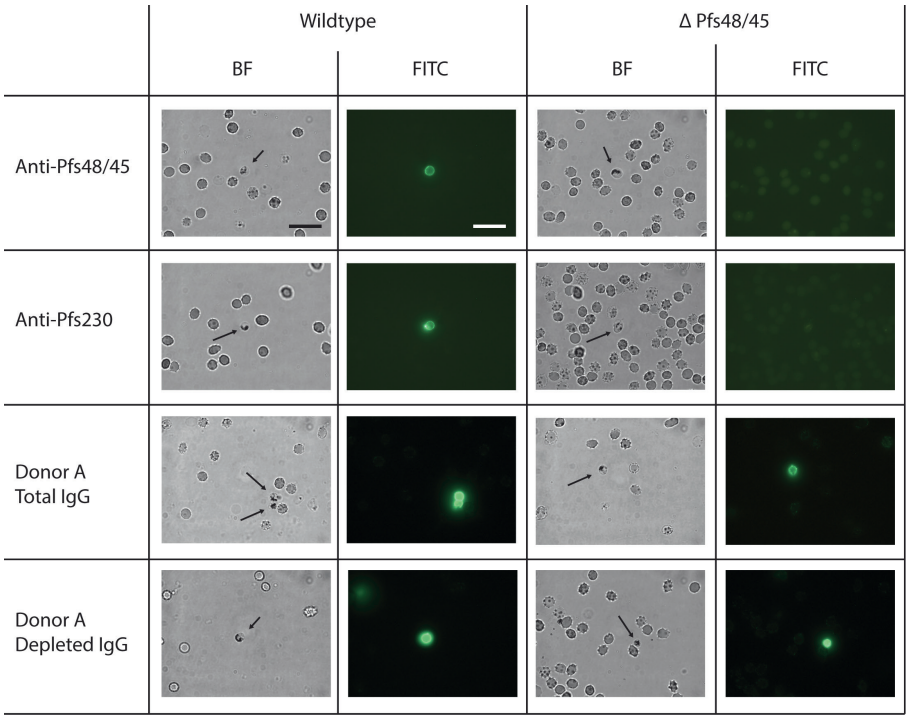


Figure 4. Gamete surface immuno-fluorescence assay (SIFA) using wild-type and Pfs48-45 KO NF54 gametes, with Pfs48/45 mAb, Pfs230 mAb, and IgG from a malaria exposed serum donor. Donor IgG is from donor A in table 3, and was performed using total IgG, and IgG depleted of α-Pfs48/45-10c and α-Pfs230CMB Ab. Δ Pfs48/45 = Pfs48/45 KO [3]. BF = Bright-field, FITC = fluorescein isothiocyanate. Anti-Pfs48/45 is mAb 45.1, and anti-Pfs230 is mAb 2A2, as described in the methods. Scale bar is 20μm.

Antibody responses to a number of the 43 novel proteins that are associated with TRA (**Table 2**) may be biomarkers of high prior gametocyte exposure rather than causally related to TRA. We thus curated this list of proteins by excluding genes with known or predicted intracellular products, while retaining genes based on evidence for a role in transmission reduction or gamete viability, or predicted compatibility with gametocyte/gamete surface expression. In addition to Pfs230 and Pfs48/45, 13 novel proteins possess sequence characteristics that are compatible with surface expression or have known roles in gamete viability (**Table 4; Supplementary Data 4** and may thus form biologically plausible targets of transmission-inhibiting antibodies. mAbs specific to Pf11-1, one of the biomarkers with a role in gamete egress, were shown previously to reduce transmission to mosquitoes in membrane feeding experiments [58]. Previous data show that the *P. berghei* homologue of another of our markers, PF3D7_1449000 (PfGEST), shows partial association with gamete surfaces and has a clear role in gamete egress [59]. We tested three monoclonal antibodies

(mAb) against PfGEST in the SIFA and SMFA. SIFA with PfGEST mAbs showed no, or very limited surface reactivity, indicating that PfGEST is not retained on the gamete surface by 20 minutes after erythrocyte egress in *P. falciparum* (**Supplementary Fig. 5B**). Though two antibodies produced modest TRA in singlicate SMFA experiments at 10 μg/mL (Generalised linear mixed model (GLMM): maximum 49.3% TRA, 95% CI 31.0-62.8, p<0.001), this was not replicable and showed no improvement with concentration (**Supplementary Fig. 4**). These data indicate that though naturally acquired PfGEST Ab appear to be biomarkers of TRA, there is no current evidence to support a mechanistic role in TRA.

The 13 potentially surface expressed proteins were represented by 39 targets on the microarray, of which 16 were statistically significantly associated with TRA in the SMFA. Though antibody responses to most gametocyte proteins decrease with age, responses to these 16 targets shared a pattern more similar to Pfs48/45 and Pfs230; breadth of response to these targets increased with age (p=0.007) while magnitude of response, though highest in older individuals, showed no age-dependent association (**Supplementary Fig. 6**). Gametocyte positive individuals recognizing ≥4/16 of these targets (4 being the 75th percentile of breadth of response to these targets) showed lower infectiousness (LogR: OR 0.36 [95% CI 0.23-0.56], p<0.001) and infection rates (OR 0.32 [95% CI 0.16-0.62, p=0.001) in the DMFA (**Fig. 3 A & B**). These patterns remained significant when Pfs48/45 or Pfs230 seropositive individuals were excluded (infectiousness: LogR, OR 0.34 [95% CI 0.20-0.58], p<0.001; infection rate: OR 0.31 [95% CI 0.15-0.64], p=0.001).

Table 4. Proteins with TRA associated antibody responses

Biomarkers of TR immunity, and plausible or known gametocyte/gamete surface proteins							
Gene ID	**Gene description**	**TM**	**SP**	**Parasite stage**	**Exported**	**Intracellular**	**Cellular component [homology/annotation]**
PF3D7_0209000	6-cysteine protein (P230)	0	Y	Gametocyte specific			known gamete surface protein, complex with P48/45
PF3D7_1346700	6-cysteine protein (P48/45)	1	Y	Gametocyte specific			known GPI anchored gamete surface protein
PF3D7_0305300	conserved Plasmodium membrane protein, unknown function	11		Gametocyte enriched, shared			extracellular region, membrane [transmembrane transporter]
PF3D7_1021100	conserved Plasmodium protein, unknown function	2		Gametocyte specific			membrane
PF3D7_1038400	gametocyte-specific protein (Pf11-1)	1		Gametocyte specific			host cell cytoplasm, implicated in gamete egress
PF3D7_1107900	mechanosensitive ion channel protein	5		Gametocyte enriched, shared			membrane
PF3D7_1143700	conserved Plasmodium protein, unknown function	1		Gametocyte enriched, shared			cytoplasm, membrane
PF3D7_1306500	MORN repeat protein, putative	10		Gametocyte enriched, shared			N/A (MORN, unknown cell component)
PF3D7_1314500	cop-coated vesicle membrane protein p24 precursor, putative	2	Y	Asexual enriched, shared			integral component of membrane
PF3D7_1360500	guanylyl cyclase beta (GCbeta)	21		Gametocyte enriched, shared			N/A (GCbeta, unknown cell component)
PF3D7_1433200	conserved Plasmodium protein, unknown function	1		Gametocyte enriched, shared			integral component of membrane, plasma membrane
PF3D7_1449000	gamete egress and sporozoite traversal protein, putative (GEST)	0	Y	Gametocyte specific			endoplasmic reticulum, microneme, osmiophilic body, implicated in gamete egress
PF3D7_1014300	conserved protein, unknown function	8	null	Gametocyte enriched, shared			cytoplasm, membrane
PF3D7_1348000	conserved Plasmodium protein, unknown function	2	null	Gametocyte specific			cytoplasm, microtubule
PF3D7_1324600	conserved Plasmodium protein, unknown function	3		Gametocyte specific			cytoplasm, membrane

Table 4. Continued

Biomarkers of TR immunity, not plausible gametocyte/gamete surface proteins							
Gene ID	**Gene description**	**TM**	**SP**	**Parasite stage**	**Exported**	**Intracellular**	**Predicted component/function [homology]**
PF3D7_0102200	ring-infected erythrocyte surface antigen (RESA)	0		Asexual enriched, shared	Y		host cell plasma membrane, merozoite dense granule, symbiont-containing vacuole
PF3D7_0801000	Plasmodium exported protein (PHISTc), unknown function	1		Asexual specific	Y	Y	host cell cytoplasm
PF3D7_0220000	liver stage antigen 3 (LSA3)	2		Asexual enriched, shared	Y		membrane
PF3D7_0702300	sporozoite threonine and asparagine-rich protein (STARP)	0	Y	Asexual enriched, shared			cell surface
PF3D7_0936400	ring-exported protein 4 (REX4)	1		Asexual enriched, shared	Y	Y	host cell cytoplasm
PF3D7_1104700	RNA polymerase subunit, putative	0		Gametocyte enriched, shared			DNA-directed RNA polymerase III complex
PF3D7_1149200	ring-infected erythrocyte surface antigen	0		Asexual specific	Y	Y	N/A (RESA)
PF3D7_0301700	Plasmodium exported protein, unknown function	2		Asexual enriched, shared	Y	Y	Maurer's cleft [Cytochrome-c551]
PF3D7_0306400	FAD-dependent glycerol-3-phosphate dehydrogenase, putative	1		Gametocyte specific		Y	glycerol-3-phosphate dehydrogenase complex, mitochondrion
PF3D7_0322600	conserved Plasmodium protein, unknown function	3		Gametocyte enriched, shared		Y	nucleus
PF3D7_0323100	conserved Plasmodium protein, unknown function	1		Gametocyte enriched, shared		Y	integral component of membrane, intracellular
PF3D7_0505000	conserved Plasmodium membrane protein, unknown function	4		Gametocyte enriched, shared		Y	N/A [Dos2-interacting transcription regulator of RNA-Pol-II]
PF3D7_0523400	DnaJ protein, putative	2		Asexual enriched, shared	Y	Y	N/A [DNA-J and DNA-J-X domains]
PF3D7_0603600	conserved Plasmodium protein, unknown function	2		Asexual enriched, shared		Y	nucleus
PF3D7_0628200	protein kinase PK4 (PK4)	1		Asexual enriched, shared		Y	N/A [two protein kinase domains]
PF3D7_0726400	conserved Plasmodium membrane protein, unknown function	8		Gametocyte enriched, shared		Y	cytoplasm
PF3D7_0731800	alpha/beta hydrolase, putative (GEXP08)	1	Y	Gametocyte enriched, shared	Y	Y	apicoplast [hydrolase]
PF3D7_0804500	conserved Plasmodium membrane protein, unknown function	8		Gametocyte enriched, shared		Y	membrane [Rav1p_C; RAVE pro (assembly of vacuolar ATPases)]
PF3D7_0815300	FAD-dependent monooxygenase, putative	1	Y	Asexual enriched, shared		Y	N/A

Table 4. Continued

PF3D7_0930500	diacylglycerol kinase, putative (DGK1)	2		Gametocyte enriched, shared		Y	apicoplast
PF3D7_1103800	CCR4-NOT transcription complex subunit 1, putative (NOT1)	4		Gametocyte enriched, shared		Y	CCR4-NOT complex
PF3D7_1212100	peripheral plastid protein 1, putative (PPP1)	1	Y	Asexual enriched, shared	Y	Y	apicoplast, host cell cytosol
PF3D7_1354200	inositol-polyphosphate 5-phosphatase, putative (IP5P)	3		Gametocyte enriched, shared		Y	N/A (IP5P)
PF3D7_0826100	E3 ubiquitin-protein ligase, putative	2		Gametocyte enriched, shared		Y	N/A (PPP1)
PF3D7_0922100	ubiquitin-like protein, putative	2		Gametocyte enriched, shared		Y	N/A (E3 ubiquitin-protein ligase)
PF3D7_1010700	dolichyl-phosphate-mannose protein mannosyltransferase, putative	0	Y	Asexual enriched, shared		Y	endoplasmic reticulum membrane, integral component of membrane
PF3D7_1127500	protein disulfide isomerase, putative	1	Y	Gametocyte specific		Y	endoplasmic reticulum lumen, membrane
PF3D7_1326500	conserved Plasmodium protein, unknown function	1		Gametocyte enriched, shared		Y	cytoplasm, cytoskeleton [FAST kinase-like protein, subdomain 1]
PF3D7_0830600	Plasmodium exported protein (PHISTc), unknown function	1		Asexual enriched, shared	Y	Y	Maurer's cleft
PF3D7_1024800	conserved Plasmodium protein (update: exported protein 3), unknown function	2	Y	Gametocyte specific		Y	integral component of membrane, symbiont-containing vacuole membrane [MMACHC, Update: Exported protein 3]

TM	Transmembrane domains (TMHMM: Plasmo DB, PMID1115261. Credits Anders Krogh, Bjorn Larsson, Gunnar von Heijne, and Erik L.L. Sonnhammer).
SP	Signal peptides (SignalP: PlasmoDB. PMID15223320. Credits Bendtsen JD, Nielsen H, von Heijne G, Brunak S).
Parasite stage	Gametocyte score category based on an analysis of previous gametocyte and asexual proteomic databases, as indicated in methods[53]. Details in Supplementary Data 1 and 4.
Exported	Exported during gametocytogenesis (table 2, Silvestrini 2010, PMID: 20332084)
Intracellular	Predicted to be intracellular, or surface expression not excluded by gene ontological terms, empirical data, or homology (see Supplementary Data 4)
Predicted/known cellular component [homology]	Gene ontological terms (curated or computed) from Plasmo DB version 28, or annotation for previously characterised proteins. For proteins without annotation or predicted function/location, domain prediction based on protein homology is presented in square brackets where available (HHPred protein prdomain prediction, The MPI bioinformatics Toolkit as an integrative platform for advanced protein sequence and structure analysis, Alva V, Nam SZ, Söding J, Lupas AN. Nucleic Acids Res. 2016 Jul 8;44(W1):W410-5)

Discussion

In this study, we assessed antibody-mediated transmission reducing activity (TRA) in individuals naturally exposed to *P. falciparum* and identified associated antibody responses. A minority of samples (3.3%) demonstrated strong and reproducible levels of TRA in controlled *in vitro* assessments with cultured *P. falciparum* gametocytes; high-level TRA was associated variably with antibody responses to gametocyte antigens Pfs48/45, Pfs230, and 43 newly described proteins. These antibody responses were associated with reduced mosquito infection rates in feeding assays with local mosquito species on infected, gametocytaemic individuals. Whilst not all of the identified antibody responses will be causally involved in TRA, we provide the first direct evidence for independent roles of antibodies specific to Pfs48/45 and Pfs230 in naturally acquired TRA. The TRA of mAb specific to one of the novel TRA biomarkers, PfGEST, indicate that PfGEST antibodies elicited by *P. falciparum* exposure are likely to be non-functional biomarkers of TRA.

The transmission of *P. falciparum* to mosquitoes is influenced by numerous factors, including gametocyte density [60], sex-ratio [61], and human host factors that are currently poorly characterised. A direct effect of naturally acquired antibodies on transmission efficiency is suggested by the observation that mosquito infection rates generally increase in mosquito feeding experiments on naturally infected gametocyte carriers when autologous plasma is replaced by control serum [62]. Antibody responses to Pfs48/45 and Pfs230, established target antigens for transmission blocking vaccines [15, 36], are naturally acquired upon gametocyte exposure and have previously been statistically associated with TRA as measured in the SMFA [10, 21-28]. The strength of these associations is variable and sometimes absent [29]; TRA is often observed in individuals without measurable α-Pfs48/45 or α-Pfs230 antibodies [10, 23, 25, 28, 30, 31]. We performed SMFA experiments on purified IgG samples from 648 malaria-exposed donors and confirm previous associations between TRA and α-Pfs48/45 (OR 4.4, 95% CI 1.5-12.9) and α-Pfs230 antibodies (OR 7.9, 95% CI 2.8-22.8). Using a unique sample set with transmission data from memDMFA from three endemic settings, we further provide the first evidence that α-Pfs48/45 and α-Pfs230 antibodies are associated with reduced transmission efficiency during natural infections. Both the likelihood of infecting any mosquitoes and the proportion of infected mosquitoes were significantly lower for individuals with α-Pfs48/45 and α-Pfs230 antibodies. For the first time, we formally demonstrate that naturally acquired antibodies to these gametocyte antigens can independently inhibit mosquito infection after affinity purification.

Despite the important role of antibodies to these two pre-fertilization antigens, 45% of plasma donors with high-level TRA had no measurable α-Pfs48/45 or α-Pfs230 antibodies, and we observed significant levels of TRA after IgG from a selection of donors was depleted of α-Pfs48/45 and α-Pfs230 antibodies. Our affinity purification experiments were based on the binding of antibodies to epitopes present in the Pfs48/45-10C and 230-CMB vaccine

constructs, since the proteins' complex conformational structures so far preclude their production as full-length proteins. We thus captured the most potent transmission-reducing antibodies (Pfs48/45 epitopes 1-3 [21, 22, 36], Pfs230 epitopes within the C region [15]) but not necessarily all antibodies to these proteins. Monoclonal antibodies against epitope 5, which would not bind to the Pfs48/45-10C protein, have been associated with weak TR activity [63]. However, given their lower efficacy and the immuno-dominance of epitopes 1-3 in naturally immune sera [21] it is highly unlikely that α-Pfs48/45 antibodies to epitopes other than epitopes 1-3 would have contributed considerably to TRA. This is also supported by the finding that antibodies to epitope 5 are only observed in the presence of antibodies to epitopes 1-3 [21]. Because all known α-Pfs230 antibodies are complement dependent [64, 65], whilst TRA in the antibody-depleted fraction is complement independent, a role for residual α-Pfs230 antibodies (responsive to epitopes outside the aa. 444-730 region of Pfs230 represented by the 230CMB product) is similarly unlikely. Crucially, IgG depleted of antibodies to Pfs48/45 epitopes 1-3 recognized surface antigens of a knockout parasite line that fails to express Pfs48/45 and Pfs230, formally demonstrating recognition of other target antigens by immune sera. Our findings of TRA in the absence of measurable α-Pfs48/45 or α-Pfs230 antibodies, together with persisting recognition of gametes that do not express these two proteins, and functional TRA after depleting IgG of antibodies to these two targets, thus strongly suggest that responses to other antigens may contribute to naturally acquired TRA.

Using a protein microarray, we aimed to identify whether responses to other gametocyte targets were associated with TRA. This array was enriched for putative gametocyte surface antigens but surface expression or gametocyte specificity were not pre-requisites for inclusion on the array. Our aim was to determine an immune signature of TRA that could improve our understanding of transmission efficiency from natural infections. We therefore included markers of gametocyte exposure as well as general markers of recent malaria infection. We observed that responses to 43 proteins were statistically significantly associated with high-level antibody-mediated TRA. Responses to these antigens were strongly predictive of transmission efficiency from natural *P. falciparum* infections with diverse gametocyte strains and locally relevant mosquito species. Whilst further studies are needed to assess the generalisability of this signature of TRA across endemic settings, the strong association of our SMFA-based signature of TRA with reduced transmission potential during natural infections is very promising. Future studies should aim to assess the kinetics of antibody responses to these antigens and their association with TRA using longitudinal cohorts, with well-defined clinical, parasitological, and infectivity data.

The microarray proteins were produced in an *Escherichia coli* based *in vitro* transcription-translation (IVTT) system. As conformation is not verified for all proteins, those requiring post-translational modifications will likely not have been expressed in their native conformation. This was exemplified by Pfs48/45 that has a known complex structure [36, 66] and showed minimal reactivity or discriminative power when printed on the array as an

IVTT product (**Supplementary Data 2**). This apparent disadvantage of the IVTT system will plausibly have affected our results for other conformational proteins, so the list of proteins in **Table 3** must be treated as a 'rule in' rather than 'rule out' selection for proteins associated with antibody mediated TRA.

Among the proteins we identified as having TRA associated antibody responses, 13 novel proteins have sequence characteristics suggestive of surface expression and may be targets of TRA. Previous data indicate that two of the proteins found to be biomarkers of TRA in our analysis, PfGEST and Pf11-1, were previously shown to have important roles in gamete egress from the erythrocyte in mosquitoes [58, 59]. Interestingly, Pf11-1 mAbs are able to reduce transmission, possibly by disabling full gamete egress [58]. Our SIFA data with humanized mAbs indicate that PfGEST has no clear surface expression in *P. falciparum* 20 minutes after activation, which may explain their lack of replicable TRA in mosquito feeding assays compared to similar concentrations of α-230 specific mAb. As there is only a narrow window of opportunity for αPf11-1 and αPfGEST antibody binding during gamete egress, activity against gamete surface proteins with roles in fertilization is a more biologically plausible mode of effective antibody-mediated TRA. Future experiments may identify which of the 13 other TRA associated plausible antibody targets in **Table 4** are expressed at the gamete surface. Our findings identify the further characterisation of functional and non-functional gametocyte antibody responses in individuals with naturally acquired TRA as a research priority. Whilst the prevalence of high-level TRA decreases with age, an opposite trend is observed for the magnitude and prevalence of antibodies to Pfs48/45 and Pfs230 and to newly identified gametocyte antigens, in spite of their overall association with TRA (**Supplementary Fig. 6)**. A better characterisation of these antibody responses in relation to functional activity is needed and may include a detailed assessment of antibody isotype, affinity and complement-binding activities.

Regardless of their functionality, our data indicate that possession of antibody responses to a group of *P. falciparum* proteins increases the likelihood of antibody mediated transmission inhibition. Combined with assessment of α-Pfs48/45 and α-Pfs230, this signature of TR immunity may improve our understanding of natural malaria transmission dynamics.

Methods

Study participants

Serum was collected from individuals recruited to epidemiological studies in Burkina Faso, Cameroon and the Gambia (**Table 1**), as well as from Dutch migrants with a history of residence in *P. falciparum* malaria endemic regions, who reported having been diagnosed with clinical malaria infection. Details of sample collection and recruitment criteria have been published for all samples from individuals living in endemic areas, but are provided

here as a summary. Parasitological status was defined by microscopy acknowledging that this lacks the sensitivity to detect many low-density infections; i.e. microscopy negative samples were not treated as an unexposed control.

Cameroon: Samples (n=140) were collected between 2011 and 2015 from individuals aged 5-16 years, during surveys at primary schools in the Mfou district, 30 km from Yaoundé. Data from these individuals has in part been presented previously [42, 43], but the remainder were provided by Isabelle Morlais (IRD, Montpelier) without prior publication. Participants were primarily *P. falciparum* gametocyte carriers identified among asymptomatic children by microscopical screening. Gametocyte density (median [IQR]=30 [16/80]/μL, among gametocyte positives) was calculated against 1000 leukocytes, assuming a density of 8000 leukocytes per μL.

Gambia: Samples (n=226) were collected from individuals resident in Farafenni and nearby villages, approximately 200 km east of Banjul, in 1992-1994 [24, 44], 1998 and 1999 [45], 2001[46, 47], and 2002 [48]. Individuals were aged 1-17 years at the point of sampling. Participants were enrolled if they were found to be asymptomatically infected with malaria parasites, based either on the observation of gametocytes [24, 44], or asexual parasites [45-48] by microscopy. Gametocyte density (median [IQR]=63 [10/390]/μL, among gametocyte positives) was either calculated by examination of 100 high powered fields, assuming that 1 gametocyte per field equated to 500/μL (threshold of 5 gametocytes/μL) as in Greenwood & Armstrong 1991[67] , or by calculation against 1000 leukocytes, assuming a density of 8000 leukocytes per μL.

Burkina Faso: Samples were collected from residents in the villages of Laye and Dapélogo (n=192), approximately 30km northeast and north of Ouagadougo, in the Central Sudan savannah area [37, 40, 41], and from residents of Soumousso and Dande (n=33), villages close to Bobo-dioulasso [38, 39]. The age of study participants ranged from 1.8-74 years. Recruitment was either random [37, 41], or based on the observation of gametocytes [38-40] or asexual parasites [40] by microscopy. Gametocyte density (median [IQR]=64 [32/128]/μL, among gametocyte positives) was calculated against either 500 or 1000 leukocytes, assuming a density of 8000 leukocytes per μL.

Netherlands: Dutch individuals were recruited by clinical staff at Radboud university medical centre in Nijmegen, the Netherlands, and had either recently returned (<6 months) from extended periods in endemic areas (n=8) or had returned more than a year before sampling (n=49). Age ranged from 26-84 years. Those who had returned more than a year before sampling had lived in areas with endemic *P. falciparum* transmission for longer than 15 years and reported between 2 and >80 clinical malaria episodes during that time. For these individuals malaria infection history was only available through verbal testimony, whereas 3 out of 8 of the recently returned migrants had confirmed recent or current malaria infection. Samples were all collected between 1994 and 2014. As detailed previously, sera from some of these expatriates show strong and consistent TRA in the SMFA [68].

All samples were collected after written informed consent was obtained from participants and/or their parent(s) or guardian(s). Ethical clearance was provided by the National Ethics Committee of Cameroon, the Gambian Government/Medical Research Council Joint Ethics Committee, the Ethical Review Committee of the Ministry of Health of Burkina Faso and the Centre MURAZ ethical review committees, the London School of Hygiene & Tropical Medicine ethics committee and by the Radboud University Medical Center ethical committee.

Sample preparation

For SMFA, IgG was purified from all plasma samples using Protein G HP Spintrap (GE Healthcare, GE Uppsala, Sweden), and concentrated to the original serum volume using Vivaspin 20 centrifugal concentration columns (Sartorius AG, Goettingen, Germany) following the manufacturer's instructions. Antibody purification was only attempted for samples with sufficient serum volume for duplicate SMFAs (180 μL). For the serological assays, an additional 30 μL of serum was retained if sample volume permitted. Because of the high serum volumes necessary for the antibody depletion/purification experiments, these were performed on only six samples with transmission blocking antibodies. Three of these six were processed by protein microarray. IgG for depletion/purification was purified from 1mL of serum using protein G high performance affinity columns (HiTrap™ Protein G HP 1 mL, GE Healthcare, Uppsala, Sweden), and then adjusted to the original serum volume for testing in the specific Pfs48/45 and Pfs230 affinity columns using Vivaspin 20 centrifugal concentration columns (Sartorius AG, Goettingen, Germany). 300 μL was immediately removed for SMFA and serological assays, leaving 700 μL of total IgG at physiological concentration for affinity column purification. Purification was repeated as required to allow for antibody eluate concentration.

Direct membrane feeding assay (DMFA)

DMFA was performed during the original studies for 498/579 individuals from endemic areas, as described in the separate study protocols using identical glass mini-feeders [54] (Coelen Glastechniek, Weldaad, the Netherlands) and the same experimental protocol [69]. Of the 498 individuals with DMFA data, 494 had microscopy based estimates of asexual and gametocyte density; only gametocyte positives (n=366) were used in our DMFA analysis. All sites used colonies sourced from eggs caught in local water sources. Colonies were of *A. gambiae s.s.* (>90%) (The Gambia [24, 44-48]), *A. coluzzii* (Cameroon [42, 43] and Bobo Dioulasso in Burkina Faso [38, 39]) or a comprised a mixture of A. *coluzzii*, *A. gambiae s.s.* and hybrid forms (other Burkina Faso sites [37, 40, 41]). Locally adapted mosquito colonies from all sites are available upon request. Data presented in the current manuscript are the proportion of infectious individuals and percent of mosquitoes infected with oocysts after feeding on whole blood [69].

Standard membrane feeding assay (SMFA)

Mosquitoes used for the assessment of antibody TRA in the SMFA were three to five-day old female *An. stephensi* (Sind-Kasur Nijmegen strain) [70], which were reared at 30°C and 70-80% humidity while exposed to a 12 hour day/night cycle. Mature (stage IV-V) *P. falciparum* gametocytes (either NF54[54], or transgenic NF54HT-GFP-Luc [71], depending on the preferred read-out) were obtained from an automated tipper system (0.3-0.5% gametocytes, 2% haematocrit) and prepared with packed red blood cells as previously described [9, 54]. Both gametocyte strains can be obtained from the MR4 Malaria Research and Reference Reagent Resource Center (https://www.beiresources.org/MR4Home.aspx). For the preparation of mosquito blood meals containing test antibodies, 90 µL of serum IgG was added to 90 µL of freeze-dried foetal calf sera (FCS) and diluted into 35 µL human serum containing active complement. This antibody/serum mix was added to the gametocyte/red blood cell mix to a final volume of 270 µL, and kept at 37°C until it was provided to mosquitoes in the membrane feeding apparatus. Antibodies were therefore provided to mosquitoes at approximately 0.66* their physiological concentration (assuming a 1:1 plasma/cell ratio). Each SMFA run was performed to test up to 24 antibody samples simultaneously, including two control mosquito groups (against which the TRA of test samples was calculated). For control samples 90 µL of freeze-dried FCS was dissolved in 90 µL milliQ and added to the same gametocyte/red blood cell mix (270 µL total volume) as was used for the other feeds the run.

Prior to blood feeding, male mosquitoes were separated from females by aspiration. Unfed and partially fed female mosquitoes were removed from their resting containers after blood feeding. Fully engorged mosquitoes were maintained at 26°C and 70-80% humidity. Mosquito infection status was determined differently for the NF54 and NF54HT-GFP-luc parasite strains. For experiments with the native NF54 strain, an average of 19.5 mosquitoes (range 7-22) were dissected at day 7 post infection (PI). Midguts were stained in 1% mercurochrome and oocysts were counted by expert microscopists. For experiments with the transgenic NF54HT-GFP-luc strain, mosquitoes were killed on day 8 PI by freezing, and 20 were homogenised for each experiment in two pools of 10. Mosquito homogenates were well mixed and three samples per pool were assayed for bioluminescence by lysis, and addition of luciferase assay substrate using a Luciferase assay kit (Luciferase assay system [E1501], Promega, USA), according to the manufacturer's instructions and as previously described [9].

For experiments with the native NF54 strain, TRA was analysed as the percent difference in oocyst number between test and control mosquitoes. For experiments with the transgenic NF54HT-GFP-luc strain, TRA was calculated as the average percent difference in luminescence intensity for two pools of 10 mosquitoes between test and controls mosquitoes, as described [9]. TRA <0% was observed, but was generally within the normal range due to natural variation in oocyst intensity between mosquito groups. For individuals with TRA

<0%, median TRA was -41.8% (IQR -80.9 - -16.5-). For experiments with replication (e.g. the affinity purification experiments) 95% CI around the TRA estimate were calculated from luminescence or microscopical readouts for multiple feeds using generalised linear mixed models (GLMMs) [72]. Choice of readout (and therefore of gametocyte strain) varied over the time that the SMFA's were being performed, and makes no difference to the integrity or interpretation of TRA, only to its efficiency [9]. ≥90% reduction in oocyst/luminescence intensity in test mosquitoes relative to controls was classified as evidence of high-level TRA, which previous data indicate is highly replicable [68]. Samples from all individuals with >80% TRA were tested in duplicate to ensure robustness of the functional phenotype and the mean value of duplicate experiments was used to classify individuals as having high level TRA (>90% average reduction) or no evidence of TRA (<10% average reduction).

Recombinant protein production for serological assays

Recombinant, correctly folded proteins were used for antibody depletion/purification experiments and for enzyme-linked immunosorbent assays (ELISA). For all assays assessing Pfs230 specific antibodies, we used the transmission blocking vaccine candidate 230CMB (developed and purified by Fraunhofer USA Center for Molecular Biotechnology), which corresponds to amino acids 444-730 of the Pfs230 protein, and comprises the protein's pro-domain and the first of fourteen cysteine motif domains [15]. For assays with Pfs48/45, two protein constructs were used. For antibody depletion/purification, we used the chimeric R0-10C protein [36], which comprises 10 cysteines spanning epitopes 1-3 of the Pfs48/45 C-terminal domain, fused to the GLURP R0 domain [36, 40]. Antibodies specific to GLURP R0 have no effect on *Plasmodium* transmission to mosquitoes (**Supplementary Table 2**) [9]. For non-functional serological assays (ELISA, Microarray), the 10C protein was cleaved from the R0-10C as described previously [40].

Monoclonal antibodies

SMFA data using mAb specific to GLURP R0, Pfs48/45 (85RF45.1), Pfs230 (2A2) and PfGEST are presented in the **Supplementary information**. Full details for GLURP R0 and Pfs48/45 (85RF45.1) mAb production and SMFA is provided in Stone et al. 2014 [9]. Details of Pfs230 mAb production and SMFA are presented in Roeffen et al. 1995 [65]. PfGEST mAbs were produced by the Hypothesis-driven Pre-erythrocytic Antigen Target Identification Consortium (HPATIC). Briefly, HKL v2.0 Kymice^{TM} containing human variable region genes (Kymab) were immunized with recombinant protein expressed in the wheat germ cell-free system (CellFree Sciences) adjuvanted with Montanide ISA 720. Non-antigen selected splenocytes were sorted into 96 well plates, one cell/well, to generate single cell mRNA material (Atreca). Atreca's Immune Repertoire Capture (IRC^{TM}) provides Single-Cell mRNA Barcode-Sequencing with high throughput and accuracy. This platform

captured full length sequences, enabling detection of somatic mutations across the entire antibody variable region. Natively paired heavy and light chain IgG variable regions were analyzed, and constant region isotype assignments were made which were then produced via gene synthesis and recombinant expression as fully human antibodies (LakePharma). Three mAbs were tested in duplicate SMFA titration experiments. SMFA was conducted according to previously published protocols [9]. In all mAb based SMFA, the endpoint was oocyst quantification by manual dissection.

Pfs48/45 and Pfs230 antibody purification

To purify antibodies specific to the Pfs48/45 and Pfs230 proteins from transmission-reducing sera, affinity columns were created by coupling 1mg of R0-10C or 230CMB to N-hydroxysuccinimide (NHS)-activated high performance affinity chromatography columns (HiTrap™ NHS-activated HP 1 mL, GE Healthcare, Uppsala, Sweden); coupling efficiency was >80%.

For the extraction of antibodies from human serum, antibodies were extracted first using the R0-10C column, and then using the 230CMB column, as follows. The R0-10C column was equilibrated with 2mL of binding buffer (PBS (1x) before 700 µL of total IgG (at physiological concentration, fraction 1 in **Table 2**) from transmission blocking sera was added. The flow-through was collected and the column washed with 12mL of PBS. 5mL of elution buffer (0.15 M NaCl, 0.1 M Glycine/HCL, pH 2.5) was applied to the column for the collection for R0-10C specific antibodies. After elution, the purification procedure was repeated using the flow-through from the first purification, and the antibody eluates from both runs were pooled. The final flow-through and combined antibody eluates (fraction 2 in **Table 2**) were concentrated to 700 µL using Vivaspin 20 centrifugal concentration columns (Sartorius AG, Goettingen, Germany). 300 µL of the flow-through was removed for SMFA (fraction 3 in **Table 2**) and serological assays, and the remaining 400 µL was processed over the equilibrated 230CMB column as for the R0-10C column, except that the final flow through's (fraction 5 in **Table 2**) and eluates (fraction 4 in **Table 2**) were concentrated to 400 µL. Depletion of specific antibody in the flow-throughs of each column was confirmed with ELISA. Antibodies (from eluates or flow-troughs) were processed in the SMFA in two biological replicates, as for total IgG with >80% TRA in the larger SMFA screen. For 3 samples, larger starting volumes were used to allow antibody eluates to be concentrated nine times, to examine the effect of specific concentrated antibody on transmission reducing activity.

The possibility that TRA from the column fractions was due to cytokines or reactive ROx/RNOx species, which in circumstances of inflammatory crisis can inhibit parasite development in mosquitoes [73], was excluded both by our use of protein G binding columns for IgG purification for all samples, and our use of 30kDa filters for antibody concentration prior to mosquito feeding.

Surface immuno-fluorescence assays (SIFA)

The Pfs48/45 knock-out (KO) NF54 *P. falciparum* line was produced and characterised by van Dijk et al. [3] and subsequently assessed by Eksi et al. [12]; Eksi et al. showed that Pfs230 is produced but not retained on surface of Pfs48/45 disruptant gametes. Wild-type (WT) and Pfs48/45 KO gametocytes were generated using standard protocols, as described above. Culture media Pfs48/45 KO line was supplemented with 2μM pyrimethamine. Briefly, gametocytes were allowed to activate at room temperature for 20 minutes (for mAb SIFA) or 1 hour (for human IgG SIFA) in the presence of foetal calf serum (FCS), and gametes were prepared for microscopy by washing with PBS supplemented with 0.5% FCS and 0.05% sodium azide. Antibodies were added to gamete preparations in the same PBS/FCS buffer; mAbs at 5μg/ml; human IgG/sera at dilutions of 1:20. Secondary antibodies were Alexa Fluor TM 488 goat anti-human IgG (H+L), Alexa Fluor TM 488 chicken anti-mouse IgG (H+L), and Alexa Fluor TM 488 goat anti-rat IgG (H+L); all were added at dilutions of 1:200. All gamete preparations were incubated with secondary antibody at room temperature for 1 hour.

ELISA

Antibodies specific to the Pfs48/45-10C and Pfs230 (230CMB) proteins were quantified in the ELISA exactly as described previously [40]. Optical density (OD) values were normalised between assay plates by adjustment relative to a consistent point in the linear portion of a standard curve of serially diluted highly reactive human sera. Cut-offs for positivity were determined from normalised OD values using maximum likelihood methods to define Gaussian populations of low and high responders as described previously [74]. For Dutch migrants who had returned from endemic areas ≥1 year before sampling, a seropositivity threshold was calculated as the mean + 3 standard deviations of the averaged normalised OD values of eight naïve European control sera.

Protein microarray

The selection of proteins to be cloned and printed on the arrays was made primarily based on observed expression by stage V *P. falciparum* gametocytes in a single recent proteomic analysis [52]. To ensure that the array included most potential antibody targets, we preferentially included proteins that had transmembrane domains, signal peptides, or GPI anchors. To ensure that the majority of proteins that were highly abundant or 'specific' to mature gametocytes were included on the array, we used mass spectrometry data from the analysis of the stage V gametocyte proteome [52], alongside proteomic data from purified trophozoites [1] and schizonts (**Supplementary Data 5,** PRIDE accession number: PXD008250) generated in the same laboratory. Gametocyte and asexual stage-specific expression datasets were generated in relative expression values to compare protein abundances (label-free quantitative [LFQ] values) between the stages, which are presented in **Supplementary Data 1** as a fold change value.

We chose to also include proteins that were potential markers of gametogenesis identified by Silvestrini and colleagues [1]. These proteins we identified as being expressed primarily by early gametocytes, or shared between stages (proteins for 32/59 were present in the stage V database), but some were more abundant in mature gametocytes or other parasite life stages (**Supplementary Data 1**). In addition to these markers, we included four putative markers of asexual parasite exposure: PF3D7_1036000 (Merozoite surface protein 11 [MSP11]), PF3D7_0711700 (erythrocyte membrane protein 1, PfEMP1 [VAR]), PF3D7_0731600 (acyl-CoA synthetase [ACS5]), and PF3D7_0423700 (early-transcribed membrane protein 4 [ETRAMP4]), based on work by Helb and colleagues [51].

Details of the 315 proteins included on the array are given in **Supplemental Data 1**. Proteins with reference sequences longer than 1000 amino-acids were split into multiple fragments (overlaps of at least 17 a.a) for cloning, *in vitro* protein expression and printing. Because the basic criteria for the selection of proteins for microarray construction was based on data generated from a single laboratory, we cross-referenced our protein list against a cross-study analysis of gametocyte specificity [53]. In short, the specificity of any protein for the gametocyte stage was scored by determining how often it had been detected across 11 proteomic analyses (listed in **Supplementary Data 1**: 3 of gametocytes only, 3 of asexual stages only, and 5 of both asexuals and gametocytes). Proteins were binned from low to high abundance and weighted according to the retrieval rates of proteins in two curated lists of "gold standard" gametocyte and asexual genes, consisting of genes that are known to be specific for either asexual stages (n=45) or gametocytes (n=41). High expression of gametocyte gold standard proteins with concurrent absence of non-gametocyte gold standard proteins resulted in a high gametocyte score, calculated from the fraction of retrieved gametocyte genes over retrieved non-gametocyte genes. All scores were log transformed and summed over all datasets. Gametocyte scores were categorized using the scores' distribution of the gametocyte gold standard as follows: Scores above the first quartile (9.69) were considered gametocyte specific. Proteins scoring at least as high as the lowest scoring gametocyte gold standard representative (-2.46) were considered as enriched in gametocytes. Proteins with lower scores are not specific to a certain life stage and have some evidence for expression in gametocytes. All proteins scoring lower than the median of the asexual gold standard (-18.98) were considered asexual specific. This analysis confirmed that 228 of the 315 proteins had consensus evidence for enrichment in gametocytes (scores >-2.46), while 284/315 had conservative evidence for expression in gametocytes (peptides present in at least one gametocyte proteomic database, score > -10). 312/315 were present in at least one gametocyte proteomic database, giving rise to a score >-18.93. Scores for all proteins on the array are included in **Supplementary Data 1**. To avoid further curation and potential bias, no proteins were excluded prior to analysis.

Proteins were expressed using an *in vitro* transcription and translation (IVTT) system, the *Escherichia coli* cell-free Rapid Translation System (RTS) kit (5 Prime, Gaithersburg,

MD, USA). A library of partial or complete CDSs cloned into a T7 expression vector pXI has been established at Antigen Discovery, Inc. (ADI, Irvine, CA, USA). This library was created through an *in vivo* recombination cloning process with PCR-amplified CDSs, and a complementary linearized expressed vector transformed into chemically competent *E. coli* was amplified by PCR and cloned into pXI vector using a high-throughput PCR recombination cloning method described elsewhere [50]. Each expressed protein includes a 5' polyhistidine (HIS) epitope and 3' hemagglutinin (HA) epitope. After expressing the proteins according to manufacturer instructions, translated proteins were printed onto nitrocellulose-coated glass AVID slides (Grace Bio-Labs, Inc., Bend, OR, USA) using an Omni Grid Accent robotic microarray printer (Digilabs, Inc., Marlborough, MA, USA). Each slide contained 8 nitrocellulose "pads" on which the full array was printed, allowing eight samples to be probed per slide. Microarray chip printing and protein expression were quality checked by probing random slides with anti-HIS and anti-HA monoclonal antibodies with fluorescent labelling. Each chip contained 28 IVTT negative control targets (for data normalisation), and 48 IgG positive control targets (for quality control).

For analysis of antibody reactivity on the protein microarray, serum samples were diluted 1:200 in a 3 mg mL^{-1} *E. coli* lysate solution in protein arraying buffer (Maine Manufacturing, Sanford, ME, USA) and incubated at room temperature for 30 min. Chips were rehydrated in blocking buffer for 30 min. Blocking buffer was removed, and chips were probed with pre-incubated serum samples using sealed, fitted slide chambers to ensure no cross-contamination of sample between pads. Chips were incubated overnight at 4°C with agitation. Chips were washed five times with TBS-0.05% Tween 20, followed by incubation with biotin-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:200 in blocking buffer at room temperature. Chips were washed three times with TBS-0.05% Tween 20, followed by incubation with streptavidin-conjugated SureLight P-3 (Columbia Biosciences, Frederick, MD, USA) at room temperature protected from light. Chips were washed three times with TBS-0.05% Tween 20, three times with TBS, and once with water. Chips were air dried by centrifugation at 1,000 x g for 4 minutes and scanned on a GenePix 4300A High-Resolution Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA). Target and background intensities were measured using an annotated grid file (.GAL).

Raw microarray protein target and local background fluorescence intensities, target annotations and sample phenotypes were imported and merged in R (Foundation for Statistical Computing, Vienna, Austria), where all subsequent procedures were performed. Foreground target intensities were corrected for local background using the backgroundCorrect function of the *limma* package [75]. Next, all corrected values were transformed using the base 2 logarithm. The dataset was normalised to remove systematic effects by subtracting the median signal intensity of the IVTT controls for each sample. Since the IVTT control targets carry the chip, sample and batch-level systematic effects,

but also antibody background reactivity to the IVTT system, this procedure normalises the data and provides a relative measure of the specific antibody binding to the non-specific antibody binding to the IVTT controls (a.k.a. background). With the normalised data, a value of 0.0 means that the intensity is no different than the background, and a value of 1.0 indicates a doubling with respect to background.

Data analysis

Quality control plots were made after each treatment of the microarray data, which included boxplots and density plots of probe intensity by study sample and probe type. Recombinant Pfs230 CMB was printed on the array for comparison to ELISA with the same protein, yielding a correlation co-efficient (Spearman's rank) of 0.44 ($p<0.001$). A universal seropositivity threshold for reactivity to array proteins was established as the IVTT background plus 3 standard deviations, which equated to a $\log_2$-transformed signal intensity threshold of 0.92 (reactivity was therefore defined as approximately twice that of the background). Because high-level transmission-reducing activity was uncommon in our sample set (22/649 samples had TRA ≥90%), antigens were defined as 'reactive' if they elicited seropositive responses in at least 1% of the study population (≥7 subjects), and unreactive antigens were included in all analyses. The universal cut-off was used only for defining proteins as reactive or unreactive, and for depicting antibody breadth for all microarray proteins.

Statistical analysis was conducted using R (Foundation for Statistical Computing, Vienna, Austria) or STATA 12 (StataCorp., TX, USA). The magnitude of antibody reactivity to each antigen on the microarray by individuals in different groups was tested by empirical Bayes moderated T-tests [76], and by logistic regression after data binarisation using protein specific cut-offs; cut-offs were generated using maximum liklihoods methods (which determines the junction between two Gaussian signal distributions for each protein that proteins cut off point for positivity) [74]. For the latter, proteins were dis-included from the analyses if the mixture models failed to stably converge, or had to restart more than once. In both the Bayes and logistic models, p-values were adjusted to control the false discovery rate below 5% using the Benjamini-Hochberg (BH) method [57], and a finding was considered significant for FDR controlled p-values (q-values) less than 0.05. For analysis of TRA on a continuous scale, TRA was used as the log transformed relative intensity, to normalise the data and avoid compression at 0%. For clarity, figures presenting TRA on a continuous scale present the linear TRA with a base of 0%. Linear models with adjustment for gametocyte density (determined by microscopy, and given as gametocytes/μL) were used to test for differences in continuous variables between groups. Logistic models adjusted for gametocyte density were used to test for the association of binary variables between groups, presenting odd-ratios and 95% CI. Differences in the magnitude of response to all proteins by groups of individuals (in contrast to the Bayes analysis, which

compares responses to individual proteins by different groups of individuals) were assessed with t-tests or ANOVA. Differences in mosquito infection prevalence in the DMFA were analysed with logistic models adjusted for gametocyte density, while differences in mosquito oocyst prevalence were assessed with logistic models, adjusted for gametocyte density and with host (individual the mosquitoes were feeding upon) as a random effect. All box-plots are standard Tukey's whisker and box plots, with Tukey's method for outlier determination. All DMFA based analysis was performed on gametocyte positive individuals only. Protein domain predictions for proteins with TRA associated responses were made using Inter-pro [77] or HhPred protein domain prediction [78].

Data availability

The novel *P. falciparum* Schizont mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [79] with the dataset identifier PXD008250. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information, have been deposited in the DRYAD data depository (doi:10.5061/dryad.8bp05), or are available from the authors upon request.

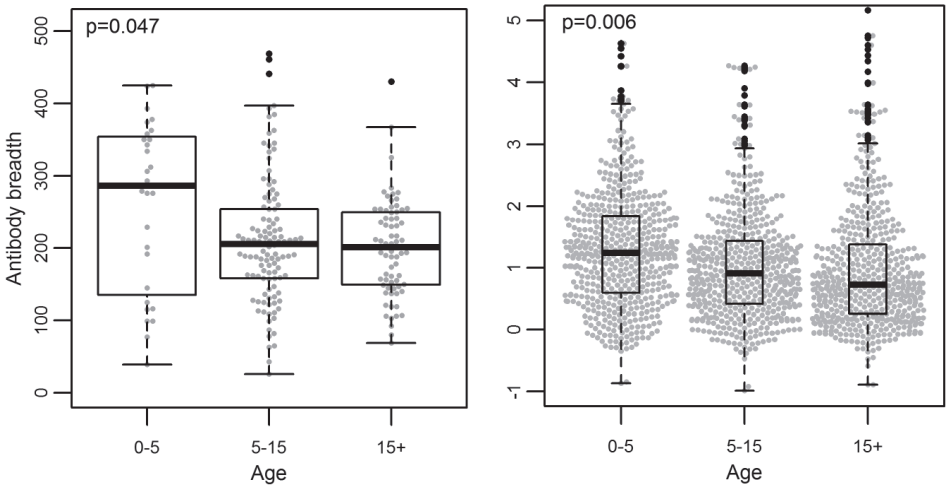
Acknowledgments

This work was supported by a Marie Curie Career Integration Grant from the European Community's Seventh Framework Programme (SIGNAL, PCIG12-GA-2012-333936), and by a VIDI fellowship from the Netherlands Organization for Scientific Research (NWO; project number 016.158.306). Data collection in Burkina Faso was supported by the Bill and Melinda Gates Foundation (AFIRM OPP1034789).

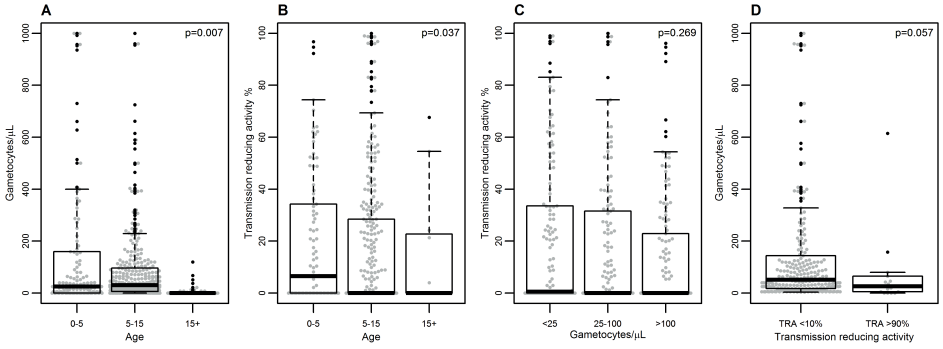
PfGEST mAb were produced by the Hypothesis-driven Pre-erythrocytic Antigen Target Identification Consortium (HPATIC) and provided by PATH's malaria vaccine initiative.

We thank the participants of the various trials contributing samples and data to this study, and the staff involved in these trials. We thank Jacqueline Kuhnen, Laura Pelser-Posthumus, Astrid Pouwelsen, Jolanda Klaassen, Maarten Eldering, and Tonke Raaijmakers for their role in sample preparation, mosquito breeding, and conduct of the SMFA. We thank Sanna Rijpma for her assistance with the preparation of samples for proteomic analyses. We thank Kassie Dantzler and Siyuan Ma for their assistance with the microarray analysis. We thank Guido Bastiens for arranging sampling of Dutch migrants, and the missionaries for their agreement to participate in the current study by donating blood for serological and functional analyses. We also thank Jessica Chichester (Fraunhofer) for providing us with Pfs230CMB.

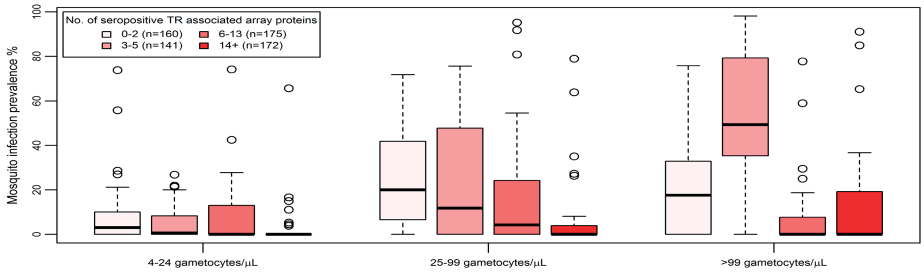
Supplementary materials



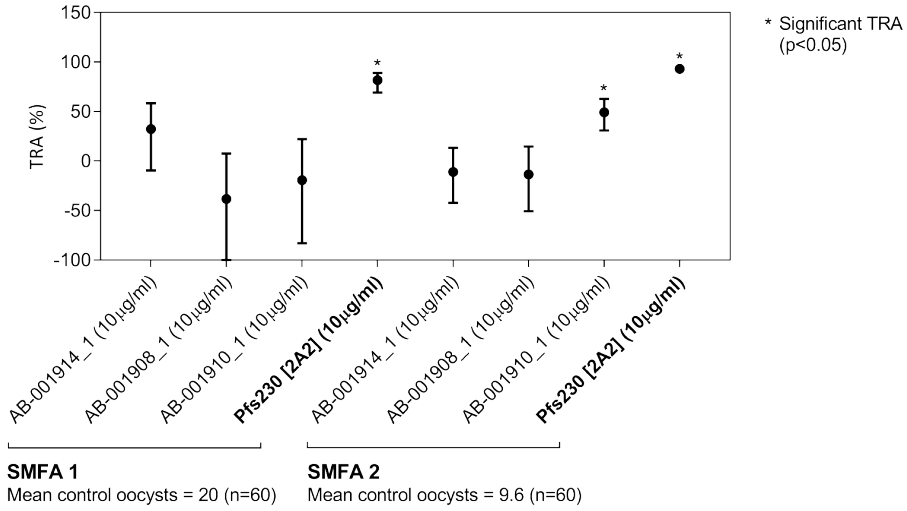
Supplementary figure 1. Antibody responses to the gametocyte protein microarray, with age data for Burkina Faso only. Responses by age are for individuals from Burkina Faso only, which was the only endemic country with substantial numbers of recruits from all age groups. Antibody breadth is the number of proteins reactive above background in each individual. Antibody magnitude is the log2-transformed average signal intensity (SI) for each microarray target, minus the vehicle SI. This equates to the log2-fold change over background. Responses are shown to each protein target by all individuals within given groups**.** For all box-plots, outliers are shown in black while all data points are shown in grey as a bee-swarm.



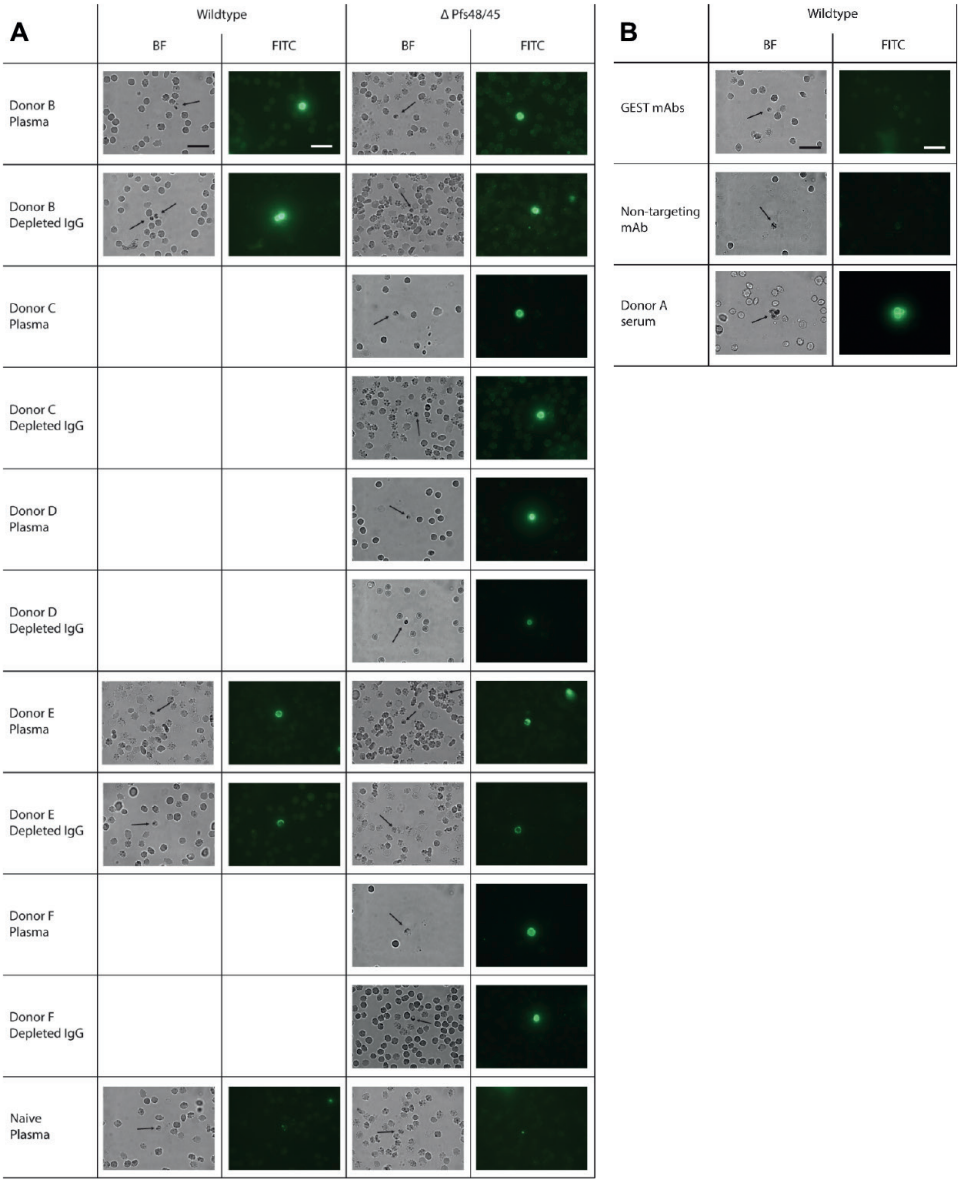
Supplementary figure 2. Gametocyte density and transmission reducing activity. Gametocyte density and TRA over age (**A** & **B**) are for individuals living in endemic areas only. Gametocyte density is shown to 1000 gametocytes/µL in **A** and **D**. Values above this limit are excluded from the plots (points missing: 0-5=7, 5-15=12, 15+=0), but not the summary statistics for the box-plots (median, IQR). TRA on a continuous scale (**B** & **C**) is presented as absolute TRA for clarity, but the p values are from simple linear regression using log relative infectivity (log[oocysts in treatment mosquitoes/oocysts in control mosquitoes]), to normalise the data and avoid compression at zero. All plots and statistical analysis with age or gametocyte density include only individuals from endemic areas. Grouped TRA (**D**) is presented for individuals with antibodies causing TRA (TRA) ≥90% vs gametocyte positive individuals with antibodies causing TRA <10%, as presented elsewhere. For all box-plots, outliers are shown in black while all data points are shown in grey as a bee-swarm.



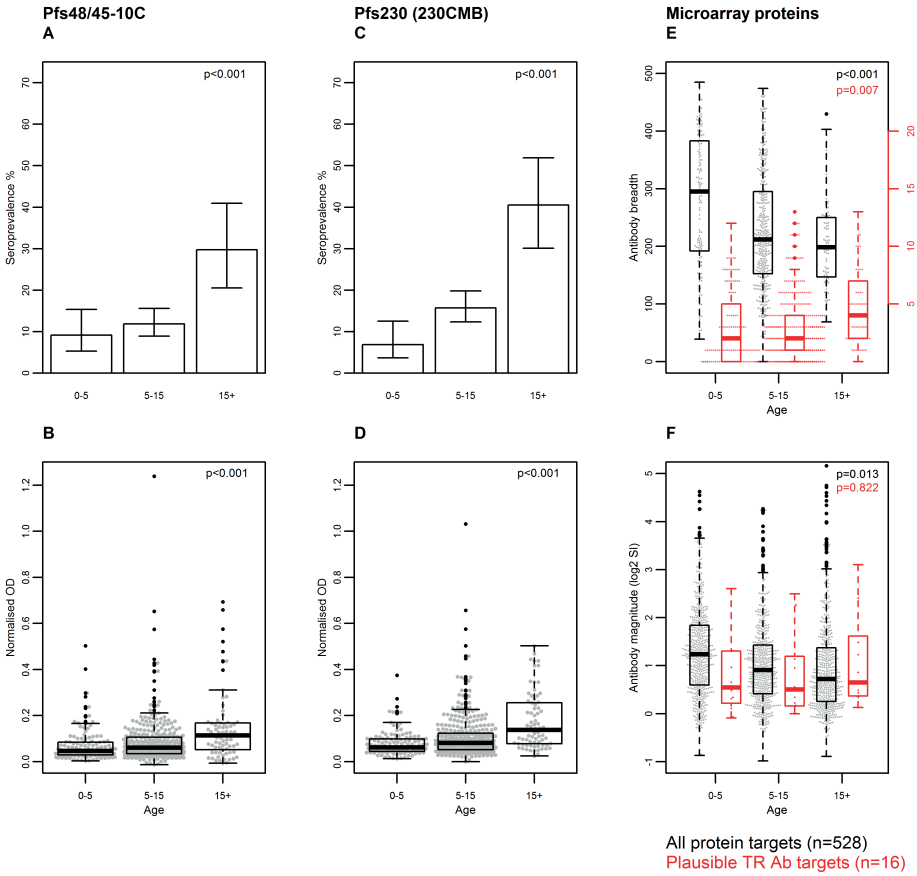
Supplementary figure 3. Mosquito infection prevalence decreases with breadth of response to novel TRA associated protein across all gametocyte densities. Breadth of response to the 61 novel TRA associated protein targets from the array analysis were binned into quartiles among the population with DMFA data (n=366). Box plots show the median, 25th, 75th 5th and 95th percentiles of mosquito infection prevalence. For all box-plots, outliers are shown as hollow circles.



Supplementary figure 4. TRA of mAbs against Pfs48/45, Pfs230, and novel biomarker PfGEST. For Pfs230 and PfGEST mAB, TRA was calculated against isotypic negative control mAb (*α-CSP*). Pfs48/45 TRA was calculated using human serum as negative control. Details for Pfs48/45 (85RF45.1) mAb production and SMFA is provided in Stone et al. 2014 [9]. Details of Pfs230 mAb production and SMFA are presented in Roeffen et al. 1995 [65]. PfGEST mAb were produced by the Hypothesis-driven Pre-erythrocytic Antigen Target Identification Consortium (HPATIC) and provided by PATH. SMFA was conducted according to previously published protocols [9]. In all mAb based SMFA, the endpoint was oocyst quantification by manual dissection. Between 20 and 60 mosquitoes were dissected in each experiment. P-values are from a generalised linear mixed model (GLMM), as described previously [72].



Supplementary figure 5. Gamete surface immuno-fluorescence assay (SIFA) using wild-type and Pfs48-45 KO NF54 gametes, with PfGEST mAb and IgG/plasma from a malaria exposed serum donor. Donors B and E and naïve donor plasma are described in table 3. SIFA was performed with human antibody sources from whole plasma or total IgG depleted of α-Pfs48/45-10c and α-Pfs230CMB Ab. Δ Pfs48/45 = Pfs48/45 KO [3]. BF = Bright-field, FITC = fluorescein isothiocyanate. GEST mAbs were a mixture of 5μg/mL of all three GEST mAbs described in the methods. Scale bar is 20μm. **A.** Donor sera, **B.** mAb, with serum for comparison.



Supplemental figure 6. Figure 1. Antibody responses to Pfs48/45-10C, Pfs230 and to the microarray proteins with age, with microarray targets that are plausible antibody targets indicated. Antibody responses to conformational, recombinant Pfs48/45 and Pfs230 were measured using ELISA, with antibody intensity given as the ELISA optical density (OD) values (450nm), which is normalised between assay plates by adjustment relative to a consistent point in the linear portion of a standard curve of serially diluted highly reactive human sera. Antibody responses to microarray proteins are given as the log_2-transformed signal intensity (SI) minus the vehicle SI, which equates to the log_2-fold change over this background. All graphs show only individuals from endemic areas (Dutch migrants excluded). Sample size: 0-5 = 131, 5-15 = 366, 15+ = 71. **A & C:** Bars show the seroprevalence of α-Pfs48/45-10C and α-Pfs230 antibodies with age, with Clopper-Pearson confidence intervals. **B & D:** Box plots showing α-Pfs48/45-10C and α-Pfs230 antibody intensity with age. **E**: Box-plots showing responses to the microarray proteins (n=528), and the targets which were identified as having characteristics which make surface expression or involvement in gamete viability possible (n=16). As indicated in the text, these 16 targets represent fragments of 13 unique proteins. Antibody breadth is the number of proteins reactive above background in each individual, within the given groups. **F:** Magnitude of antibody response to microarray proteins and plausible Ab targets. Each spot represents the average SI of response to each protein target by all individuals within given groups. P-values for prevalence data are from likelihood ratio test for differences in seroprevalence between all age groups, derived from logistic regression, and adjusted for gametocyte density. P values for intensity data and response breadth are from an F test for differences in OD/SI between all age groups, derived from linear regression, and adjusted for gametocyte density, or from students t-test (for magnitude only). For all box-plots, outliers are shown with larger black/red dots while all data points are shown in grey/red as a bee-swarm of small dots.

Supplementary table 1. Transmission-reducing activity and association with Pfs48/45 and Pfs230 antibody responses

SMFA selection		Samples	Pfs230 (230CMB)				Pfs48/45 (Pfs48/45-10C)				Either/both of Pfs230 and Pfs48/45			
			% (n/N)	OR (TRA >90%)	95% CI	p-value	% (n/N)	OR (TRA>90%)	95% CI	p-value	% (n/N)	OR (TRA>90%)	95% CI	p-value
Total	**TRA (all)**	*648*	*16.2% (105/648)*	-	-	-	*14.4% (93/648)*	-	-	-	*21.8% (141/648)*	-	-	-
Blockers	**TRA ≥90%**	*22*	*50.0% (11/22)*	-	-	-	*40.9% (9/22)*	-	-	-	*54.5% (12/22)*	-	-	-
Comparators *all samples*	**TRA <90%**	626	15.0% (94/626)	5.8	2.2-15.8	<0.001	13.4% (84/626)	4.0	1.4-11.3	0.009	20.6% (129/626)	5.1	1.9-14.0	0.001
	TRA <10%	405	15.6% (63/405)	5.5	2.0-15.3	0.001	14.6% (59/405)	3.7	1.3-10.5	0.015	22.0% (89/405)	4.7	1.7-13.0	0.003
Comparators *gct positive only*	**TRA <90%**	406	12.1% (49/406)	7.3	2.6-20.3	<0.001	11.1% (45/406)	4.7	1.6-13.6	0.004	17.2% (70/406)	6.2	2.2-17.0	<0.001
	TRA <10%	254	10.6% (27/254)	7.9	2.9-23.7	<0.001	11.4% (29/254)	4.4	1.5-13.2	0.007	16.9% (43/254)	5.9	2.19-17.45	0.001

% (n/N)	Seroprevalence of antibody responses to Pfs230, Pfs48/45, or either protein. n = seropositive individuals, N=total population size
TRA %	Transmission-reducing activity of purified IgG in the standard membrane-feeding assay (SMFA), relative to control mosquitoes fed the same gametocyte batch without test antibodies. TRA is the mean of two independent SMFA runs for all samples.
Samples	Total number of samples with TRA assessed in the SMFA
n/N	Number of samples seropositive in ELISA/Number of samples assessed in the ELISA
OR (TRA ≥90%)	Odds ratio of association of seropositivity and TRA, where the presence of TRA is defined as TRA ≥90%, and the absence of TRA is defined by the comparator groups TRA <90%, and <10%. Logistic models are adjusted for gametocyte density.
95% CI	95% confidence intervals of the OR
p-value	P-value from the logistic model used to generate the OR

Supplementary table 2. The TRA of GLURP-R0 compared to dilutions of mAb specific to Pfs48/45 epitope 1 (85RF45.1) and control human serum in the SMFA. Full details of these SMFA experiments are in Stone et al. 2014 [9].

Treatment	SMFA 1		SMFA 2		SMFA 3	
	Mean oocysts	TRA %	Mean oocysts	TRA %	Mean oocysts	TRA %
Control (human serum)	6.8	-	13.3	-	17.9	-
85RF45.1 mAb (0.15µg/ml)	6.3	7.2	11.2	15.8	30.7	-71.4
85RF45.1 mAb (0.3µg/ml)	2.9	57.0	13.6	-2.5	16.1	10.2
85RF45.1 mAb (0.6µg/ml)	1.4	79.8	7.5	43.7	9.2	48.7
85RF45.1 mAb (1.25µg/ml)	0.1	98.0	1.2	90.7	1.9	89.4
85RF45.1 mAb (2.5µg/ml)	0.1	99.0	0.4	96.7	0.3	98.5
GLURP mAb (2.5µg/ml)	6.9	-2.2	14.1	-6.0	12.1	32.5

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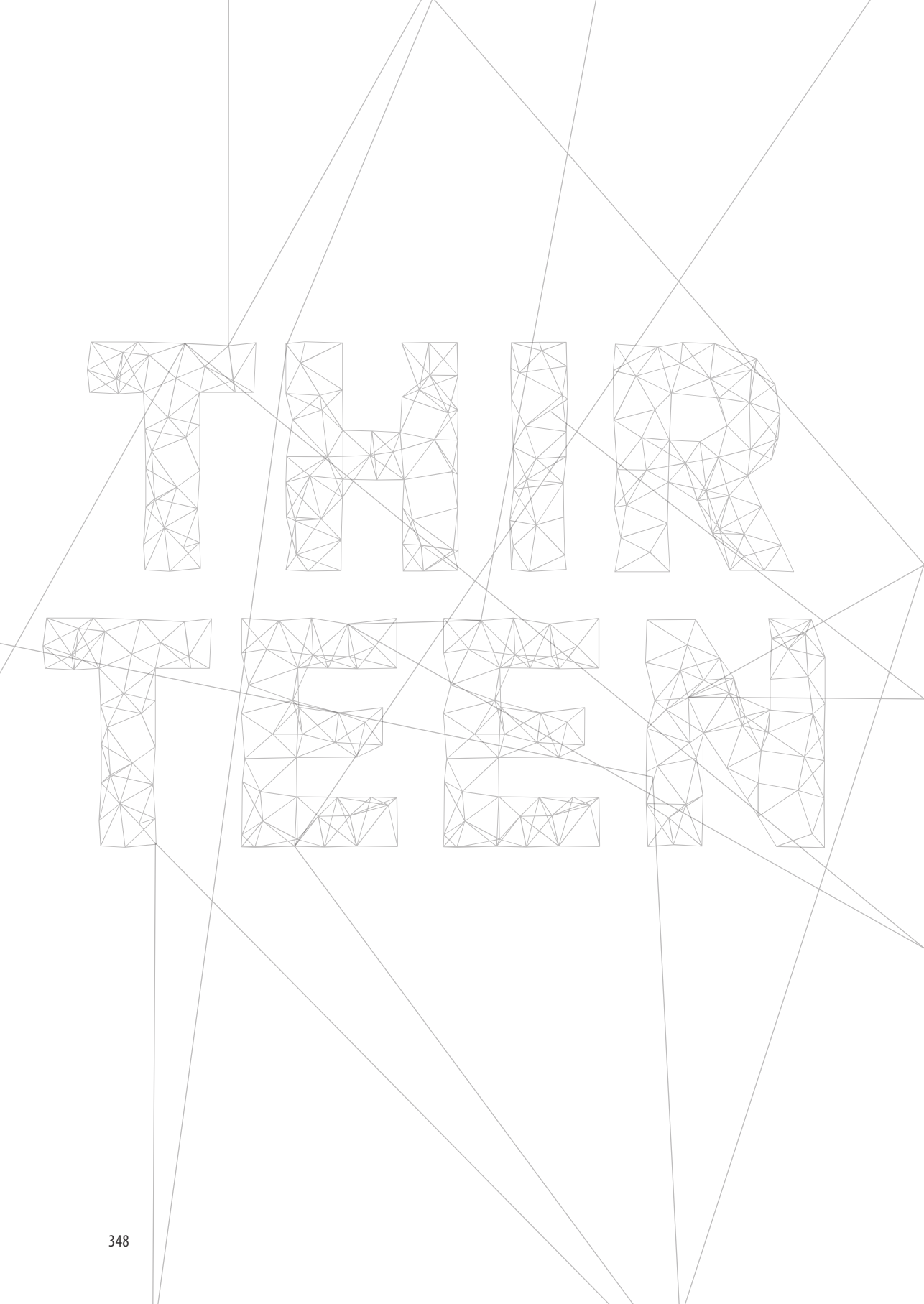
THIRTEEN

Chapter 13

Discussion

The need to characterise the malaria infectious reservoir

The aim of this thesis was to investigate the effectors of human malaria infectiousness to inform the development and application of transmission-reducing interventions. **Chapter 2** aimed to develop an operationally attractive tool to identify pockets of current and recent malaria transmission. The work was part of a larger project based on the concept that the infectious reservoir of malaria might be targeted by proxy, and that interventions focused on hotspots of high prior exposure (and therefore, high infection risk) might reduce transmission in the community at large [1, 2]. The theory underlying this is that factors like housing quality, local geography, and health seeking behaviour combine with and reinforce inequalities in vector exposure [3-5], such that the inhabitants of defined areas suffer disproportionately high incidence of infection, and sustain transmission to the wider community [6, 7]. The work in **chapter 2** dealt only with developing tools to enable this kind of population-wide screening, not on its deployment, which was part of a separate trial in Western Kenya in 2012 [8]. The proxy measures of malaria risk were the presence of current malaria infection, determined by nested PCR based detection of ribosomal or mitochondrial DNA, and cumulative exposure to malaria infection, determined as the presence of antibodies specific to the malaria blood-stage antigens AMA-1 and MSP-1 [9]. Both techniques were well known and used but our methodology made their concurrent assessment an operationally realistic pre-face to intensive control or elimination [10]. We showed that the techniques could be combined efficiently, with no significant loss of sensitivity to either the serologic or the molecular outcome. Combining the filter paper solute washing and DNA extraction into a single sample processing step meant that the ELISA and PCR procedures could be complete within 24 hours, while performing the assays on separate samples would require significantly greater pre-processing, roughly doubling the time to assay read-out.

In the absence of perfect vector controls effective against indoor and outdoor biting mosquitoes, reducing the number of infectious humans is key to reducing transmission. Without a policy of indiscriminate treatment, such as is used in China during elimination campaigns [11] and in public health emergencies globally [12], this requires that the majority of infectious individuals should be identified and treated. At its simplest, ensuring coverage of all infectious individuals can be achieved by targeting all infected individuals. The molecular techniques in **chapter 2** would be well suited to this kind of surveillance. Serology provides an invaluable measure of population level transmission intensity, and such measures are likely to be extremely useful measures of transmission reducing efficacy and elimination. However, accurately identifying the infected population at a single time point requires molecular assessment. There are clear benefits to assessing both current and historic infection during population surveillance: PCR provides the minimal unit for

targeting during active case detection, while serology (assessed at the population level) provides a measure of transmission change for program monitoring, and can indicate locations or populations with high malaria risk for presumptive control. Since many infections may be of short duration [13], it is also conceivable that many infections are missed when screening by PCR whilst a serological 'footprint' of this infection allows a more informative assessment of force of infection at an individual level. The key to the efficiency of the combined serological and molecular assessments in **chapter 2** was the use of finger-prick blood spotted onto filter paper as the source of antibodies specific to malaria parasites, and parasite DNA. Though they are a simple and efficient source of DNA, the use of dried blood-spots necessitates significant delays between infection detection and intervention [14, 15]. High volume, whole blood samples improve sensitivity, but these benefits are offset by the complex logistics of sample storage and transport. A point-of-care test with equal or higher sensitivity than dried blood spot based PCR is the ideal alternative. Loop mediated isothermal amplification (LAMP) meets these requirements; it can be used can detect parasites at densities of as little as 0.3-2.5 parasites per μl [16, 17], and requires only the tests reagents and a water bath to amplify genus and species specific DNA (variously detectable as a visible colour change or one measurable with a spectrophotometer) [18]. Conventional RDTs are also in development with comparable sensitivity to PCR [19], though the historically low specificity of these tests may remain a limiting factor [14].

Ultra-sensitive diagnostics treat all infected individuals as potentially and equally infectious, but in reality, there is likely to be significant heterogeneity in the contribution of different population members to onward transmission. Molecular assessments detecting gametocyte specific mRNA (primarily QT-NASBA, amplifying *Pfs25* transcripts) have shown that the previous assumption that gametocytes are produced by a minority of individuals [20] were an artefact of insensitive diagnosis [21-26]. There may therefore be little to gain from targeting transmission-reducing interventions to individuals based on gametocyte rather than parasite prevalence. Mosquito infection becomes probable at high gametocyte densities, but the lack of association between gametocyte density and infectivity to mosquitoes at lower densities (which are common in natural infections) make gametocyte density an unreliable indicator of infectivity. Intrinsic factors (internal factors derived from the parasite or host) may enhance the infectivity of low-density infections, or suppress the infectivity of high ones, and extrinsic factors (external environmental factors effecting the likelihood of transmission) have similar effects, increasing or annulling the possibility of infectious individuals contributing the mosquito infectious reservoir. With our current understanding of what determines infectiousness and transmission potential, assessments of infectivity are unlikely to have a direct role in control targeting. The later chapters of this thesis focus on understanding and measuring infectiousness at both the individual and population level. Understanding heterogeneities in the infectious reservoir

would facilitate better control, and may feed into targeted campaigns. For the evaluation of transmission reducing interventions, assessments of infectivity provide the most direct measure of success.

The role of mosquito feeding assays in the development, targeting, and evaluation of transmission reducing interventions

Mosquito feeding assays are the gold standard measure of human malaria infectivity, but they are not discussed in the context of surveillance and control targeting because of their operational complexity. Nonetheless, xenodiagnostic techniques (traditionally used to evaluate the presence of infectious organisms by studying their transmission to another, readily dissectible host) have essential roles in investigative epidemiology (**chapter 4**), in the development of transmission reducing drug and vaccines in the laboratory (**chapters 5**, **6 and 7**), and in the evaluation of these in endemic settings (**chapters 8** and **9**).

Delineating infectiousness and transmission potential

Population-wide use of mosquito feeding assays is uncommon, but becoming more widely advocated because of its uniquely comprehensive readout. Whether or not mosquitoes become infected when feeding on an infected host depends on an array of factors (gametocyte density, maturity, parasite strain, human immunity) and feeding assays are the only tool currently able to determine whether the host at that given time is capable of successfully infecting mosquitoes and at what rate. Our work in **chapter 4** describes the various studies that have assessed the infectious reservoir using xenodiagnosis. Our conclusion was that assessments cannot only rely on feeding assays conducted across transects of endemic populations, but must incorporate as natural as possible a reflection of variation in exposure to mosquitoes. Ultimately, the contact rate between mosquitoes and human hosts dictate the transmission potential of infectious individuals. We questioned the dogma that children, being highly gametocytaemic and therefore highly infectious, need to be targeted with greater priority than adults to interrupt transmission effectively. Not only do previous studies demonstrate that older individuals contribute substantially to mosquito infections because of their demographic abundance [27], we further suggest that differential exposure and attractiveness further polarises the contribution of different age groups. What leads from this, practically, is that no age group should be preferentially targeted by transmission reducing interventions; children are more infectious and more vulnerable to symptomatic infection so require coverage, but older individuals may sustain transmission, so interventions must cover the entire infectious population.

Rigorous site-specific assessments of the infectious reservoir are not pre-requisite to local elimination (as those in **chapter 2** may be). Instead, we suggest that their conduct in a few locations with varying patterns and intensity of transmission, preferably longitudinally, could inform elimination programs widely. It is particularly important that these assessments be conducted in areas close to elimination, as the data presented in our review and in the literature focus on areas with higher endemicity. Mosquito feeding assays conducted on this scale may be daunting, but for epidemiological studies (rather than control targeting, where this level of detail may be less important) there are no accurate alternatives. One study conducted in southern Tanzania attempted to measure human infectiousness without feeding assays, by studying infection rates in mosquitoes that were caught after feeding on sleeping individuals [28]. Blood fed-mosquitoes were collected from deliberately 'holey' bed nets, so that oocysts in the mosquito's guts could be classified according to their size, allowing prediction of the night (and therefore individual) infectious feeds occurred on. Theoretically, these experiments reflect natural infectivity better than feeding assays, because mosquito infection was the result of both human infectiousness and mosquito host choice. However, as all study participants were placed under equally poor quality nets, normal variations in exposure (as would exist if the environment were not modulated by the study) had no impact on transmission potential. Low and unpredictable mosquito feeding rates (and therefore sample sizes) are also likely to be extremely limiting to the assays utility. Primarily though, and quite rightly, these techniques would be unjustifiable on ethical grounds, even with appropriate treatment of the exposed individuals.

The roles of age and behaviour-dependent variation in mosquito exposure have thus far been ignored in assessments of the human infectious reservoir for malaria. The simplest analysis of vector exposure would be to use existing data on body size and control coverage across age groups to adjust feeding-assay based estimates of infectiousness. However, the relationship between control coverage and age will vary significantly between locations, so this post-hoc analysis is imperfect. A better but highly complex analysis would be to determine vector exposure for each person during surveillance using human and mosquito blood-meal genotyping, such as was used in Peru to demonstrate that adults were more often bitten by *Aedes aegypti* mosquitoes than children in the same houses [29]. A recent study by our own group has combined xenodiagnostic surveillance with these individual assessments of biting behaviour. In one area with intense, seasonal transmission in Burkina Faso, population adjusted infectivity data showed that children <5 years old were responsible for 25.8% of mosquito infections in the dry season, and 32.9% in the wet season (Bronner Goncalves, *personal communication*). Genotyping data from the same area showed that more than 95% of mosquito blood meals were from individuals >5 years old, meaning that after the infectivity data were adjusted for differences in vector exposure, children contributed less than 4.4% of the total number of infective blood meals. On the other hand, the contribution of older individuals (>15 years) to the pool of infected mosquitoes increased from 21.7-

22.1% to 50.3-53.9% after adjustment for exposure. In light of these data, the 'infectious reservoir' as it has traditionally been measured is rendered almost irrelevant. As patterns of vector biting behaviour, effective vector exposure and transmission intensity vary widely and may change profoundly during the scaling up of control initiatives, there is a clear need for further studies assessing infectivity and transmission potential simultaneously. As transmission decreases and residual transmission from outdoor biting mosquitoes becomes more important, these studies will have to be adapted to sample indoor and outdoor resting mosquitoes if the infectious reservoir is to be identified accurately. Population or area wide vector surveillance is labour intensive, and household level vector exposure is generally assessed with traps which sample mosquitoes as they feed and rest inside houses, or as they escape to rest outdoors [30]. Mosquitoes that feed and rest outdoors are more challenging to sample and target with vector controls, but for elimination it will be important to measure exposure to these species because of their abundance in areas with historically high levels of net and insecticide use [31-33]. These difficulties may be circumvented by assessing vector exposure serologically. Not only malaria antigens (**chapter 2**) but also mosquito antigens, egested into the bite wound in the saliva, can induce measurable antibody responses in exposed humans (**chapter 3**). [34-40]. For assessment at the population level, salivary bioassays could be made highly efficient if adapted for use with dried blood spots, using the methodology described in **chapter 2**, and have the important advantage that they may be developed for the assessment of both indoor and outdoor biting species.

Surrogates of mosquito exposure

The mosquito salivary protein gSG6 (*Anopheles gambiae* salivary gland protein 6) described in **chapter 3** was initially selected as a biomarker because of its transcript abundance, small size (10kb), specificity to mosquitoes of the *Anopheles* genus, specificity to females, and the immunogenicity of its peptides [41-44]. Our work was the first to assess the utility of αgSG6 responses as markers of heterogeneity in vector exposure at fine spatial scale. A key advantage of the gSG6 assay is that the protein appears to elicit short lived antibodies, whose presence parallels short term changes in vector abundance, such as those resulting from seasonal fluctuations [42, 45] or vector controls [44]. Though only published in part, we studied responses to gSG6 in individuals from Apac, Uganda, an area of historically intense malaria exposure, showing that decreased responses to the salivary protein accurately reflected the success of recent interventions [46]. Since the publication of our primary study, which remains the most detailed concurrent assessment of antibody responses to mosquito saliva in relation to mosquito exposure and prospective malaria risk, several new insights in SG6 homologues and resources for future work have become available. Though initially thought to be specific to the *An. gambiae* species complex, SG6 homologues have been found in *An. funestus*, *An. stephensi*, and *An. freeborni*. Antibodies specific to these proteins cross react, making αgSG6 antibodies a marker of exposure to the bites

of afro-tropical malaria vectors, rather than to those of *An. gambiae per se* [38]. Following the recent publication of the whole genomes of 16 species of *Anopheles* mosquitoes [47], specific analyses of salivary gene conservation among key malaria vectors may provide new starting points for the development of generic and specific bioassays of vector exposure [48], incorporating indoor and outdoor biting species. Future assay development will also have to focus on the dynamics of the bite response. Bite tolerance (lower antibody titre, and less severe bite inflammation) has been observed experimentally in response to repeated biting by *Culex quinquefasciatus* [35, 49], and the pattern of declining gSG6 antibody titre with age indicates tolerance also develops to *Anopheles* saliva. This affects the utility of the gSG6 bioassay, because the magnitude of response in a child will be different from that of an identically exposed adult, or between individuals with the same current but different historical biting exposure. The comparability of responses to different mosquito species may also be affected by bite toleration, as different biting frequencies could affect the rate at which the responses become tolerated. Interestingly, another *Anopheles* salivary gland protein, cE5, appears to be more immunogenic than gSG6 among all age groups, and shows no appreciable decline in response with age [50]. This biomarker may have greater utility for assessing the transmission potential of whole populations. Ideally, its informativeness would be assessed in longitudinal studies with frequent household or, in the light of the aforementioned heterogeneity in mosquito exposure, individual-level assessments of vector exposure [30]. Human landing catches may play a role here as a field standard assay for assessing indoor as well as outdoor exposure. Though labour intensive, as a proof of concept this would allow the accuracy the salivary biomarker to be set and would provide measures of the entomological inoculation rate against which surrogate biting rates can be interpreted.

Mosquito feeding assays as a surveillance tool

A major barrier to the broader use of feeding assays in surveillance is the time and skill it takes to perform the assay reliably. Key to this is the assays readout. It has been assumed that because mosquitoes appear to egest very few sporozoites into the bite wound, a mosquito with any number of salivary gland sporozoites is equally infectious [51, 52]. In **chapter 8,** we stained the remnants of ruptured oocysts in the mosquito midgut and carefully quantified the sporozoite rate in the same mosquitoes, showing that on average two thousand sporozoites were released by each oocyst. Though we showed that there was considerable loss between sporozoite release and salivary gland invasion, and that many oocysts actually failed to rupture altogether, we demonstrated that the smallest possible level of infection (single oocysts) consistently results in some level of salivary gland colonisation. These results validate the use of oocyst prevalence as a proxy for infectiousness, which is the basis on which **chapter 9** was written, which compares methods of assessing oocyst prevalence at the expense of their actual number, as a rapid read-out of human infectivity. Very recently,

data was published that challenge the initial assumption that all infected mosquitoes are equally infectious. In a retrospective analysis of controlled human and rodent malaria infection, Churcher and colleagues show that mosquitoes with heavier sporozoite loads cause infection more commonly [53]. Tens of thousands of sporozoites may establish in the salivary glands, and the small number in the mosquito inoculum (in the region of tens or hundreds) was thought to be due to the mosquito's thin salivary duct limiting the number that can pass through simultaneously [51, 54]. Churcher hypothesises that despite this mechanical restraint, variations in the time between biting, sporozoite load and sporozoite location may lead to variation in the accumulation of sporozoites in the salivary duct prior to feeding, resulting in varied sporozoite load or infectiousness [53]. These observations highlight the importance of assessing variations in human infectivity; individuals with more gametocytes are more likely to infect mosquitoes and infect heavily, and mosquitoes with more parasites may transmit more efficiently. In addition to these findings, there are also indications that sporozoite strain affects sporozoite infectivity [55]. In short, heterogeneity in the human infectious reservoir may have one more layer of complexity than was previously assumed. Though this requires consideration and merits more focused study for *P. falciparum*, it does not necessarily change what is the most appropriate outcome measure of feeding assays. Mosquito infection prevalence rather than the intensity of their infection remains the most epidemiologically relevant metric of human-mosquito transmission [56, 57], and the most conservative unit for its targeting. Because of their strong association, infection intensity in mosquitoes with low-level infections may be even be interpretable from simpler measures of oocyst prevalence [58].

The standard membrane-feeding assay

Targeting transmission starts with a robust assay for the development, prioritisation and evaluation of transmission reducing interventions. Though gametocyte infectiousness is a biological and therefore inherently variable outcome, it is essential that its assessment be standardised, to ensure validity of comparisons between compounds and vaccines during screening, and reproducibility within and between laboratories. As in the field based feeding assays in **chapters 8 and 9**, part of this standardisation may be achieved by making the denominator of infectiousness objective. In so doing, assay comparability is improved, and increases in throughput, which will be essential if feeding assays are to remain in the pipeline for intervention screening, are made possible. In **chapters 8 and 9**, this was achieved by disregarding oocyst intensity and instead determining only the prevalence of mosquito infection (and thus, leading from **chapter 8**, infectiousness), either by detection of parasite DNA using nested PCR or circumsporozoite protein by ELISA or chemi-luminescent slot-blot assay. In **chapter 5**, we developed a scalable standard membrane-feeding assay (SMFA) with an objective, luminescence-based endpoint, for the assessment of mosquito infection rate, prevalence, and for the analysis of transmission

reducing activity. Since these publications, several groups have discussed and adopted the luminescence SMFA [59-66]. The use of transgenic parasites eliminates the requirement for mosquito dissection, which requires skilled technicians, and is limited by its objectivity and speed. The difficulties in scoring oocysts are demonstrated by recent publication in which *Plasmodium vivax* oocysts had to be classified as 'oocyst like structures' due to their uncertain origin [67]. As the development of transmission-reducing drugs and vaccines receives wider academic attention, it will be increasingly important that new laboratories can efficiently detect and quantify mosquito infection, in the field and in the laboratory. Although the luminescence SMFA cannot be used to assess infectivity during natural infections, it may be used to screen libraries of sera for their transmission reducing immunity in controlled laboratory conditions (**chapter 13**). This approach could be adopted following the role-out of transmission blocking vaccines in endemic populations [68]. For this purpose, blood samples could be taken longitudinally, starting before vaccination and proceeding monthly for at least a year, but preferably for multiple transmission seasons for the assessment of immune boosting from natural gametocyte exposure. Longitudinal assessment would allow antibody half-life to be assessed, with comparison to baseline or, better, to matched unvaccinated cohorts, to account for natural seasonal fluctuations in gametocyte exposure and transmission reducing immunity.

Transmission blocking drugs

In **chapter 7**, the luminescence SMFA was adapted for use with a parasite line developed in our laboratory that was suited to indiscriminate drug screening (because it lacks the drug selectable markers of the parasite line used in **chapter 5**). We showed that certain drugs have activity that cannot be detected by standard *in vitro* assays of gametocytocidal activity, and that the broad window of drug activity that is represented in the output of the SMFA is therefore necessary for some drugs with potent transmission reducing activity. Importantly, our assay allows such drug screens to be performed at a rate 2-5 fold greater than would be possible with standard microscopical readouts. More than 6 million compounds have been tested for their activity against malaria parasites, of which around 20,000 had notable activity [69, 70]. Inevitably, the discovery rate for compounds active against asexual parasites has outstripped that for drugs active against transmission stages, because of the complexity of assessing gametocytocidal or sporontocidal activity [71]. Truly high throughput SMFA screening is and will remain out of reach, because of the required infrastructure for gametocyte culture and insect husbandry. *In vitro* assessments of transmission blocking compounds can achieve substantial increases in throughout, as demonstrated by a recent report in which 13,983 compounds were screened for their gametocytocidal activity, using a saponin-lysis based gametocyte visualisation assay [72]. Other *in vitro* assays, namely the dual gamete formation assay [61, 73, 74], interrogate a wider window of sexual developmental pathways against which drugs may be active, and can disentangle the effect

of drugs with permanent and reversible effects. As gametocytocidal activity is a more desirable characteristic than activity downstream of gametocyte activation [75], *in vitro* assays will continue to be the first step in the screening pipeline of transmission active drugs.

Primaquine represents a rare case for which most *ex vivo* screens are incapable of demonstrating its activity, which is only apparent after the drugs bio-activation in the liver by cytochrome P450 2D6 (CYP 2D6) and other enzymes [76, 77]. The work in **chapter 8** was based on the WHO recommendation that a single dose of 0.25mg base/kg PQ should be added to artemisinin combination therapy (ACT) without prior G6PD screening, for reducing *P. falciparum* transmission in areas close to malaria elimination or areas with high levels of ACT resistance [78]. The WHOs prior stance was that 0.75mg base/kg could be provided in similar areas with 'low G6PD risk' [79]. Our clinical trial in Kenya provided valuable support for combining ACT with the recommended low PQ dose to reduce gametocyte circulation time, which has until recently been lacking [80]. Unfortunately, PQ's dependence on bio-activation may further complicate its wider adoption as a gametocytocide. CYP 2D6 is highly polymorphic. Its allelic variants denote phenotypically normal, intermediate or poor substrate metabolism, and primaquine failure (relapse of *P. vivax* infection) has been linked with CYP 2D6 'poor metaboliser' (PM) status [81]. Though studies assessing CYP 2D6 allele frequency are sparse, what data there are indicate that PM alleles are uncommon in African populations (6.3%), while intermediate metabolisers (IM) are common in Africa and Asia (35-41%) [82]. There are concerns that the frequency of PM/IM alleles may affect PQ effectiveness at the recommended low dose [83]. The weight based low PQ dose for *P. falciparum* may therefore need reconsidering, but in in light of the drug's contra-indication in G6PD deficient individuals this will not be simple, and the desirability of alternative transmission blocking drugs is clear.

Methylene blue has potent gametocytocidal activity at low concentration (10mg/mL), and an excellent safety profile [84, 85]. As it is already medically licenced, its adoption is limited primarily because of difficulties producing the drug at scale. The 8-aminoquinline Tafenoquine has similar gametocytocidal activity and safety in G6PD deficient individuals as PQ [86]. However, in contrast to MB and PQ Tafenoquine has the significant advantage of being long-lasting (2 week half-life) and able to prevent sporogony in mosquitoes that ingest the drug up to 4 days after they were infected [87]. Perhaps most exciting is the novel Spiroindolone compound KAE609, that was discovered during a high throughput small molecule screen [88] and proceeded to show potent schizonticidal and gametocytocidal efficacy *in vitro*, and in the luminescence based SMFA [89]. Phase 1 and 2 trials with KAE609 have proceeded successfully, showing clearance times of *P. falciparum* and *P. vivax* in excess of ACT clearance times. Importantly, this efficacy is maintained against artemisinin resistant parasite strains [90], and studies show that the biological mechanisms of spiroindolone and artemisinin resistance are distinct [88]. The drug also appears not to

stimulate and may even prevent the emergence of dormant ring stages, which have been identified as a potential cause of recrudescence after artemisinin treatment [91]. The results of clinical trials evaluating the effectiveness of KAE609 for reducing gametocytaemia and transmission to mosquitoes in endemic populations are expected in the near future (clinicaltrials.gov: NCT02543086).

Though the results of our own trial add to the evidence that gametocytocides like primaquine can rapidly reduce the infectiousness of treated patients, the ability of such drugs to expedite transmission reduction at the population level depends on their coverage of the infectious reservoir. In this respect, the effect of achieving significant coverage with ACTs during mass administrations may overshadow the additional impact of gametocytocides [92-94]. Endectocides like ivermectin have a different mode of action and may have larger benefit as supplement to ACTs compared to primaquine [94]. The reason for this is that ivermectin has a strong but short-lived effect on mosquito mortality [95] and affects the mosquito age structure for several weeks [96-98]. Ivermectin partly circumvents the need to achieve near-universal coverage, because of the frequency of blood-feeding of anophelines during the time ivermectin is active [95, 96, 99-101]. Mathematical models suggest that endectocides have great potential as transmission reducing agents, particularly in combination with potent schizonticidal and gametocytocidal antimalarials [101].

Could we predict infectiousness without xenodiagnosis?

The later chapters of this thesis aimed to dissect the infective potential of malaria-infected individuals by considering parasite and human factors that might together determine the likelihood of infecting mosquitoes upon exposure.

Gametocytes and molecular markers of infectiousness

Principle among the intrinsic determinants of infectivity is gametocyte density. Because of their often very low densities, microscopy based surveillance usually fails to detect the majority of gametocyte reservoir. Molecular assays show that sub-microscopic infections are often infectious [102], and models indicate their relevance for transmission at the population level [103-105]. Gametocyte sex ratio may also be an important determinant of infectiousness but until recently could only be examined by microscopy in high-density infections. However, high-density infections only occur in a minority of population members, and sex-ratio is hypothesized to be most important in low-density infections [106]. Low-tech gametocyte concentration tools (magnet assisted cell sorting) are incompatible with microscopy-based sex-ratio assessments, that rely on subtle morphological discrimination based primarily on the distribution of magnetic pigments within the gametocyte [107].

Molecular assays are the only tools theoretically capable of determining not only gametocyte density, but also their sex, maturity, and other parasite factors related to infectivity.

In **chapters 10** and **11** we presented a novel gametocyte quantification assay, capable of determining the density of both male and female gametocytes over the entire biologically plausible range of infectivity. This plausible range is related to the mosquito blood meal size. If a mosquito ingests anywhere between 2-8μL of blood while feeding [52, 108], the minimum gametocyte density allowing fertilization of one female by one male gametocyte would be 0.25 gametocytes/μL. With heterogeneity in gametocyte densities in blood, potentially resulting in clustering of gametocytes within blood meals [109], the threshold of detection would ideally be lower than this. The detection limit of our assays was <0.1 gametocytes/μL. Broadly in line with this this abstract estimate of plausibility, Churcher and colleagues showed using the Pfs25 based QT-NASBA that mosquito infection probability decreased linearly below densities of 1 gametocytes/μL [110]. However, previous work indicated a puzzling association between gametocyte density and mosquito infection rates with a plateau over 1 and up to 200 gametocytes/μL where there was no obvious association between density and infection rate. This finding conflicted with smaller studies that quantified gametocytes by microscopy and suggested a positive association between gametocyte density and the likelihood of mosquito infection over this density range.In **chapter 10** we showed that when total gametocyte density is measured accurately, combining male and female gametocytes and conducting molecular assays rigorously by qRT-PCR in a single laboratory with automated nucleic acid extraction and standardized assay conditions, gametocyte density and mosquito infection rate are indeed positively correlated over this range, displaying a simple saturating relationship over the range of gametocyte densities in our study. Though gametocyte sex ratios unfavorable to fertilization success in the mosquito are uncommon, our analysis indicates that in low density infections infectivity is lower for a given female gametocyte density when fewer males are present (when male: female sex ratio was less than the median, which was approximately 1:5). This is in line with previous suggestions based on microscopy that a lower female bias may enhance infection probability when gametocytes are sparse [111, 112]. In fact, the best fit of gametocyte density and mosquito infection rate was demonstrated by modelling female gametocyte density and infection rate incorporating the estimated infectivity restriction imposed by low male densities; this fit was better than both female density alone or total gametocyte density. The low sensitivity of microscopy makes assessments of gametocyte sex-ratio impossible in most natural infections. In all mRNA based assays there is an inherent uncertainty in interpreting parasite number from transcript abundance [113]. However, ours is the first to quantify male and female gametocytes separately, with similar sensitivity, and is therefore the first to allow gametocyte sex-ratio estimation in all infections relevant for transmission. **Chapter 10** may thus provide the most accurate estimate of the association between gametocyte density and mosquito infection rates to date.

The PfMGET (Pf3D7_1469900) male marker used in our assay was discovered using RNAseq analysis of pure populations of male and female gametocytes, separated by the detection of sex-linked fluorescence markers [114]. The gene has no ascribed function at present. Interestingly, though PfMGET mRNA was abundant and near uniquely transcribed by male gametocytes, proteomic analyses by our own [114] and other groups [115] indicate extremely low or non-existent corresponding translation in mature gametocytes. Like Pfs25 then, the PfMGET protein may only be expressed during or after gametocyte activation, indicating a similarly sex-specific function. As we conducted them, the Pfs25 and PfMGET assays quantified male and female gametocytes in field samples by extrapolating their qRT-PCR signal against known quantities of the separated male and female gametocyte populations. For use with larger numbers of samples these assays may be conducted using readily available, non-transgenic, mixed sex trend-line material, for which male and female density have already been calculated. Separate male and female assays are essential for estimating sex-ratio in samples with low gametocyte densities, but as an estimate of total density the additional uncertainty introduced by having to perform separate mRNA quantifications for each sample is not ideal. To more efficiently determine total gametocyte density a molecular marker equally transcribed by both male and female gametocytes would be extremely useful. Recent transcriptomic analyses of male and female gametocytes will facilitate the search for such markers [114, 116]. Multiplex qRT-PCR assays quantifying sex-specific and pan-gametocyte markers, in addition to markers of maturity, would be the ideal molecular assays of infectiousness. These assays would ideally also be developed for *Plasmodium* species other than *P. falciparum.*

In addition to their density and sex ratio, gametocyte strain and maturity provide valuable additional measures of their likely infectivity. Neither were investigated as part of this thesis, but both will have to be considered in assays aiming to predict infectivity without xenodiagnosis. Strain specific differences in infectivity are observed in the SMFA, and may relate to generic infectivity factors (increased gametocyte formation, or per gametocyte fertilisation probability) [117] or other parasite factors interacting with species-specific mosquito factors e.g. Thioester protein 1 mediated mosquito immunity [118, 119]. Variation in gametocyte productivity may also result from human genetic factors, and has been linked with haemoglobinopothies including sickle cell trait [120] or tribal genetics (densities are lower in the Fulani than in the Mossi tribe in Burkina Faso)[121]. Variations in gametocyte productivity affect human infectivity, but not our ability to interpret infectivity from estimates of total gametocyte density. On the other hand, gametocyte maturity directly determines *per capita* gametocyte infectivity [122, 123]. Because immature gametocytes sequester, the majority of those in circulation should be mature, but variations the proportion of newly released stage IV gametocytes and the relative age of stage V's will affect the likelihood of successful transmission if overall densities are low. Molecular markers of early, mid and late gametocytes would enable infections to be characterised

according to maturity, which may explain some deviations from the relationship between gametocyte density and mosquito infection rate [26, 124]. Such markers could also adjust for the effects of host clearance efficiency on gametocyte age and infectivity. For example, an individual with many gametocytes but rapid gametocyte clearance may consistently have a greater proportion of immature gametocytes, and thus have minimal infectivity to mosquitoes, while someone with low clearance rates might have a higher proportion of mature gametocytes, and thus be more infectious. Markers of gametocyte maturity have already proved successful in an analysis of *P. vivax* infectivity, in which transcript abundance of the Pfg377 orthologue (female specific and expressed abundantly in mature gametocytes and subsequently zygotes) was positively associated with infectiousness in field based mosquito feeding assays [125]. Though further candidate markers exist, more may be revealed by assessing the transcript and proteomic development of gametocyte time series' from culture. The same techniques may identify markers responsible for variations in infectivity between different gametocyte strains.

Ideally, the expression of infectivity associated parasite factors would be assessed in longitudinal studies in areas of seasonal malaria transmission in which study participants are cleared of all infection, then monitored and sampled regularly during and following the transmission season. Gametocyte density, sex ratio, and putative markers of commitment and maturity could then be studied with reference to infectivity assessed by mosquito feeding assays conducted at several points after the initiation and treatment of blood stage infection. The molecular tools described in **chapters 10 and 11** would allow gametocyte density, sex and infectivity to be assessed with unsurpassed detail, and the samples gathered would inform the development of further infectivity associated factors.

Transmission reducing immunity

In addition to gametocyte density and fitness, human immune responses may be a decisive factor in determining the likelihood of onward transmission. Assessment of transmission reducing immune responses may have particular utility as a sero-epidemiological readout during surveillance of populations after the administration of a transmission blocking vaccine.

As discussed extensively in **chapter 12**'s review, antibody responses to the gametocyte/gamete proteins Pfs48/45 and Pfs230 do not fully explain the existence of naturally occurring transmission-reducing immunity. Specifically, antibody mediated transmission reducing immunity can be present in the absence or presence of antibodies specific to Pfs230 or Pfs48/45 [126]. Previous work has demonstrated statistical associations between functional antibody mediated transmission inhibition and antibody prevalence or density to Pfs230 and Pfs48/45 [126-135]. Whilst suggestive of a functional role of these antibodies in transmission reducing immunity, supported by the established potency of vaccine-induced rodent monoclonal antibodies against Pfs48/45 (85RF45.1) and Pfs230 (63F2A2.2a/b), formal evidence was never provided. The possibility that these responses parallel other

jointly or uniquely causal responses had thus far not been excluded. Similar confusion has been observed with antibody responses to asexual parasites, where markers of exposure mask the effects of modulators of protection from clinical infection [136, 137].

Our work in **chapter 13** makes important steps toward understanding the mechanisms of transmission reducing immunity, by definitively proving that antibodies acquired naturally to Pfs48/45 and Pfs230 can contribute to the transmission reducing phenotype. Our findings also strongly indicate the contribution of antibodies specific to unknown proteins, by showing that sera depleted of antibodies to key transmission blocking epitopes of Pfs230 and Pfs48/45 retained strong, complement independent antibody mediated transmission inhibition. Importantly, we show that responses to other gametocyte proteins are associated with transmission reducing activity (TRA), as demonstrated by antibody inhibition in the SMFA and lower infectiousness to mosquitoes in field based feeding assays. This is the first time it has been shown that antibody TRA measured in the SMFA relates to infectiousness in field settings. Specifically, individuals responsive to subsets of the newly identified antigens were considerably less likely to infect any mosquitoes (OR 0.37, 95% CI 0.24-0.25, $p<0.001$), and, if infections, infected far fewer mosquitoes (OR 0.18, 95% CI 0.24-0.55, $p<0.001$). Collectively our data are the first to prove the involvement of specific antibodies in transmission-reducing immunity, quantify the impact of this immunity on natural transmission and provide new correlates of the phenomenon that appear to predict transmission potential in multiple endemic settings.

Of the six individuals for whom we directly assessed the inhibitory activity of different antibody fractions, only one possessed transmission-blocking antibodies against Pfs48/45 (epitopes 1, 2 or 3) at sufficient physiological concentration to inhibit mosquito transmission. A number of individuals possessed lower titre responses to Pfs48/45 or Pfs230 (CMB region) that blocked after these antibodies had been concentrated. TRA in these samples is therefore either due to high titre antibody responses to other proteins (or non-protein antigens), or a combination of lower titre responses to any of Pfs48/45, Pfs230, or other targets. Unfortunately, restrictions in sample volume meant we were unable to say whether antibodies specific to the column-bound proteins, when not sufficient to block separately, were able to block when combined. The possibility of cross-antigen synergism was therefore not tested, though sero-epidemiological associations indicate such an effect may exist [138]. Pfs48/45 is GPI anchored to the gametocyte surface and is retained there during gamete activation, while Pfs230 possesses no anchor, and requires interaction with Pfs48/45 (probably mediated by the proteins C terminal domain, containing the 6 cysteine motifs which characterise the protein family) to be retained on the gametocyte/gamete surface [139]. Mutant parasites possessing disruptions in the *Pfs230* gene showed reduced binding to uninfected erythrocytes, at similar levels to parasites in which both *Pfs230* and *Pfs48/45* were disrupted [140]. Differences in the function and accessibility of the Pfs48/45 and Pfs230 epitopes bound by naturally acquired transmission blocking antibodies may alter

the functional titre of antibodies specific to both proteins when they are acting in unison. Future studies with high sample volumes, large screening populations, and longitudinal gametocyte exposure assessments would provide the necessary reagents for in depth study of the functionality of specific naturally acquired transmission associated antibodies. Another approach, becoming more realistic as the scalability of whole parasite immunisation has grown [141], would be to study the dynamics and mechanisms of induced 'natural' transmission reducing immunity by inoculating humans with inactivated gametocytes or gametes, as was done with non-human animals in the 1970's and 80's [142-145].

The antibody correlates of transmission reducing immunity identified using our protein microarray and library of transmission blocking sera may themselves be mechanistic in the transmission inhibition, or may be non-functional markers of this immunity. Expression on the gamete surface is a logical requirement for a target of transmission reducing antibodies. Though the majority of the proteins on our microarray possessed transmembrane domains or signal peptides, some may be internal and therefore inaccessible to antibody binding [146]. The list of proteins with TRA associated antibody responses provided in **Chapter 13** has therefore been divided into proteins with and without predicted or known features that exclude surface expression, to prioritise the candidates for future research. Work is ongoing to assess the potential functionality of the reported antibody responses. At present, proteins from our list of hits are being produced in a wheat germ based cell free system [147, 148], and recognition of them will be confirmed by our library of sera from transmission-reducing individuals. Subsequently, the proteins will be inoculated into rodents with a Montanide gel® adjuvant to produce polyclonal antibodies that will be tested in the SMFA later this year. This work is being funded by the Malaria Vaccine Initiative (MVI), who registered their interested in this avenue of vaccine discovery.

Transmission reducing immunity develops shortly after gametocyte exposure [135], and wanes within six months [149]. In humans [133, 150] and non-human animals where individual infections have been followed more closely [142-144], there appears to be a close association between the development of transmission reducing immunity and gametocyte density. Some of our correlates of inhibitory responses may represent biomarkers of high gametocyte density, and not have any mechanistic relevance to the development of transmission reducing immunity. To offset this possibility, comparisons of antibody association with transmission reducing immunity were restricted to gametocyte-exposed individuals, as determined by microscopy. Regardless, if gametocyte exposure increases the likelihood of developing transmission-reducing antibodies, responses associated with gametocyte exposure may predict the presence of mechanistic responses. Our immune signature may therefore have utility as a biomarker of antibody-mediated transmission inhibition that is more inclusive than the assessment of responses to Pfs48/45 or Pfs230 alone. Transmission reducing immunity was rare in our study populations, so our correlates require validation in a broader, larger selection of blockers and non-blockers, for whom exposure history and accurate molecular infection

data are available. However, it is very encouraging that the correlates or TRA identified from a subset of individuals with high inhibitory activity in the SMFA (n=22) predicted lower probability and rate of mosquito infection in the far larger population of individuals who recognised a high proportion of these proteins (n=157). This indicates not only that SMFA and DMFA are related, but that the DMFA picks up weaker but epidemiologically relevant reductions in transmission, which may be predicted by signatures of antibody response.
Protein selection for the microarray was based largely on a single proteomic analysis [114] and was restricted to only 315 gametocyte enriched proteins, while numerous recent studies indicate the presence of many more gametocyte specific proteins [115, 116, 151, 152]. Future work will involve expanded arrays, and larger, longitudinal sample sets, for assessing the dynamics and mechanism of transmission reducing immune responses. This work will be conducted in an international collaboration within the framework of cohorts of the International Centers of Excellence in Malaria Research (ICEMR).

* * *

As malaria transmission declines, the bridge between persistent low-level transmission and elimination becomes increasingly difficult to cross. Malaria elimination has been recently achieved with conventional controls and monitoring on islands [153, 154], and in areas of the Eastern Mediterranean, were transmission intensity was generally low [155, 156]. As transmission and the clinical burden of malaria decreases in African settings, elimination may be made possible or expedited by the detection and treatment of asymptomatic and sub-patent asymptomatic infections. This requires knowledge and surveillance of the infectious reservoir. Direct assessments of infectivity are essential in understanding relative contributions to the infectious reservoir. How infectiousness changes with declining transmission between different sections of the population, and between individuals with patent and sub-patent, symptomatic and asymptomatic infections, will guide decisions about wider surveillance and intervention. Transmission reduction will result in declining incidence of infection, measurable with sensitive diagnostic surveillance, and reduced rates of clinical disease, which could be assessed passively at health centres [56]. To effect the transmission reduction though, the majority of infectious individuals must be targeted, and in most areas, this will require scalable and efficient measures to detect infections and potential infectivity. More nuanced surveillance than passive case detection, or periodic surveys with insensitive rapid diagnostic tests, has been highlighted as a priority in areas where artemisinin resistant parasite strains are endemic, incorporating resistance monitoring to define the asymptomatic reservoir of resistant parasites [14, 157]. If measures of infectiousness could be incorporated into serological and molecular surveillance, they could efficiently expedite transmission reduction.

The ideal tool for assessing infectiousness would be a rapid, point of care test that determines human gametocyte infectivity and vector biting rate. Given that infectivity is not

determined by any single factor, point of care is probably out of reach, but a combination of multiplex assays with serological and molecular readouts could indicate potential or likely infectivity and prioritise areas or demographic groups based on the likelihood of exposure to vectors. Xenodiagnosis could effectively be replaced by accurate estimates of gametocyte density, sex ratio and maturity, combined with a measure of immune responses known to predict or elicit TRI. To determine which infectious individuals are likely to contribute to the infectious reservoir, transmission potential could be interpreted from an individual's repertoire and intensity of antibodies specific to range of mosquito salivary biomarkers. The relevance of low-density infections will vary between areas or households with different exposure to permissive vectors, so such information could be invaluable in areas with unpredictable transmission.

Closing remarks

Malaria transmission reduction, through interventions targeted to the infectious reservoir of parasites or the deployment of drugs and vaccines that reduce infectiousness, has never been more relevant. Considerable gains can be achieved with efficacious treatments and vector controls, but both of these at risk from resistance. The discovery of new drugs like spiroindolone KAE609 is encouraging, particularly as this was the direct result of investment in high throughput drug screening. There is hope that the efficacy of artemisinins can be prolonged by combination with new partner drugs like this one, or with multiple drugs, including gametocytocides [158]. This does not mean the search for novel treatments can stop. ACTs came into wide scale use in in the early 2000's [159], and resistance was observed within 6 years [160]. In February 2017, four individuals separately returned to the UK from Angola, Liberia and Uganda, showing reduced parasite clearance times following treatment with artemether-lumefantrine [161]. Although these individuals were treated outside research settings, leaving some uncertainties about the achieved drug concentrations in relation to treatment outcome, the observation is worrying. In the same month, an article reported a single malaria case imported to China from Equatorial Guinea, again showing slow clearance, but with extended ring stage survival in *in vitro* drug assays [162]. None of these cases showed the degree of resistance observed in the Greater Mekong region, and though the parasite imported to China had a single amino acid switch in the *Kelch 13* region, genotyping showed it was an African parasite strain. Though few in number, these cases may be the first indication that indigenous artemisinin resistance has emerged across the African continent.

As artemisinin stepped into the gap created by chloroquine and sulphadoxine-pyrimethamine resistance, so the next generation of treatments must replace artemisinins, or with careful planning and containment, be used to maintain their efficacy. To return to Lewis Carrol's Red Queen analogy, this is running to stand still. Resistance to the best available insecticides and treatments means that research must focus on finding alternatives, while

simultaneously slowing or halting their declining efficacy. If efforts to achieve sustained control require that we run at the parasites pace, then elimination requires that we run twice as fast. Conventional malaria control can be highly efficacious, but without a concerted effort to achieve elimination, it may simply turn a detectable reservoir of infection into an invisible one [157]. Targeting the infectious reservoir to achieve transmission reduction may provide the energy required to eliminate malaria, and doing this first in areas that seed the spread of drug resistance could ensure that elimination remains a feasible goal globally.

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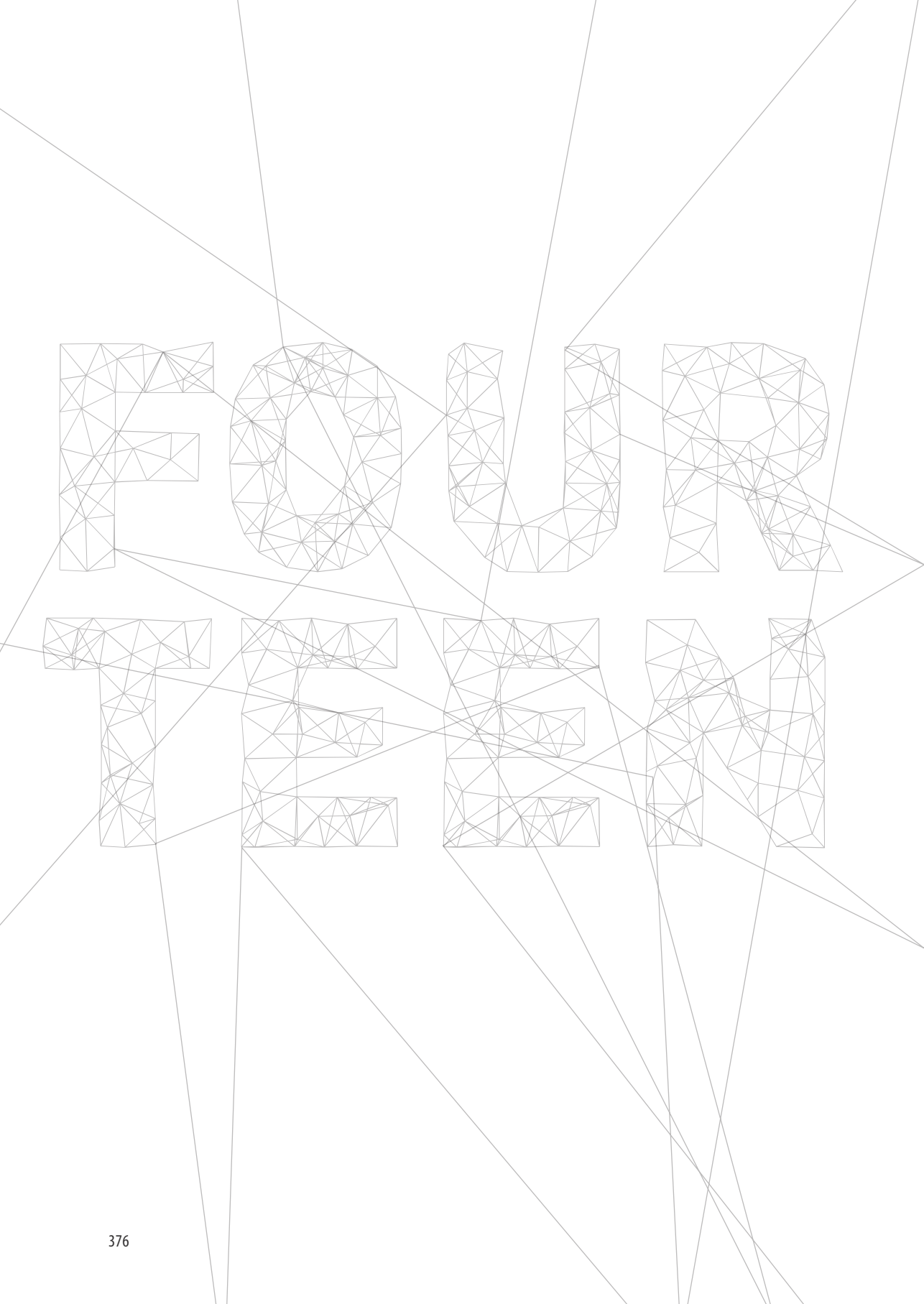
FOUR
TEEN

Chapter 14

Summary
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Summary

In spite of our best efforts, malaria remains a devastating disease. Limiting its impact depends on our ability to treat the disease effectively and to kill the parasite's vector and prevent it from biting. Efforts to eliminate the malaria causing *Plasmodium* parasites require that we can detect and understand their infectious reservoir; that is, the people within a population who transmit their infections to mosquitoes. Transmission reducing drugs and vaccines may also be required to achieve elimination, and translating their success from the laboratory to the field requires measures of *Plasmodium*'s infectivity to mosquitoes.

In Chapter 2 we developed an operationally attractive tool to identify pockets of current and recent malaria transmission, based on the detection of historical and current malaria infection. We showed that detection of *Plasmodium* DNA and human antibodies specific to the parasite could be extracted and analysed simultaneously, for use in population wide surveillance. These techniques are applicable for assessing malaria transmission at the population level, and could be used for targeting interventions to areas with high malaria exposure where, possibly, the majority of mosquitoes become infected.

Whether or not mosquitoes become infected when feeding on an infected host depends on an array of factors (gametocyte density, maturity, parasite strain, human immunity) and feeding assays are the only tool able to determine whether a host is capable of successfully infecting mosquitoes and at what rate. Our work in **chapter 4** describes the various studies that have assessed the infectious reservoir directly using feeding assays. Our conclusion was that such assessments cannot only rely on feeding assays conducted across transects of endemic populations, but must incorporate as natural as possible a reflection of variation in exposure to mosquitoes, which determines the transmission potential of individuals within the infectious reservoir.

Understanding vector exposure is complex, but it may be made simpler by assessing vector exposure serologically. Our work in **chapter 3** was the first to assess the utility of antibody responses to an *Anopheles* salivary protein, gSG6, as a marker of heterogeneity in vector exposure at fine spatial scale. An assay such as this could be used alongside infectivity data from feeding assays, or parasitological readouts, to describe heterogeneity in transmission at the population level.

A major barrier to the broader use of feeding assays in surveillance is the time and skill it takes to perform the assay reliably. Key to this is the assays readout. In **chapters 7 and 8,** we validated the use of oocyst prevalence as a proxy for infectiousness, and compared tools for rapidly assessing mosquito infection as an indicator of human infectivity in field based feeding assays. These feeding assays will be essential for the initial evaluation of transmission- focused interventions, but the development of these interventions starts in the laboratory. In **chapter 5**, we developed a scalable standard membrane-feeding assay (SMFA) with an objective, luminescence-based endpoint, for the assessment of mosquito

infection rate and prevalence, and for the analysis of transmission reducing activity (TRA). The use of transgenic parasites eliminates the requirement for mosquito dissection. In **chapter 6**, the luminescence SMFA was adapted for use with a parasite line developed in our laboratory that was suited to indiscriminate drug screening. We showed that certain drugs have activity that cannot be detected by standard *in vitro* assays of gametocytocidal activity, necessitating the retention of the SMFA in the drug-screening pipeline.

The later chapters of this thesis aimed to dissect human infective potential by considering parasite and human factors that might together determine the likelihood of malaria-infected individuals infecting mosquitoes when they are bitten. Principle among these is gametocyte density. Because of their often very low densities, microscopy based surveillance usually fails to detect the majority of gametocyte reservoir. Molecular assays show that sub-microscopic infections are often infectious, and models indicate their relevance for transmission at the population level. In **chapters 9** and **10** we used purified transgenic male and female gametocytes to develop a novel gametocyte quantification assay, capable of determining the density of both male and female gametocytes over the entire biologically plausible range of infectivity. In **chapter 9** we showed that accurately determined total gametocyte density is associated with mosquito infectivity, and that sex-ratio has an important impact on this association. In **chapter 10**, we used the assay to investigate the effect of a gametocytocidal drug, primaquine, in a new clinical trial assessing the effect of the drug gametocyte circulation in Kenyan children. The molecular tools described in **chapters 9 and 10** allow gametocyte density, sex and infectivity to be assessed with so far unsurpassed detail.

In addition to gametocyte density and fitness, human immune responses may be a decisive factor in determining the likelihood of onward transmission. Assessment of transmission reducing immune responses may have particular utility as a sero-epidemiological readout during surveillance of populations after the administration of a transmission blocking vaccine. As discussed extensively in **chapter 11**'s review, antibody responses to the gametocyte/gamete proteins Pfs48/45 and Pfs230 do not fully explain the existence of naturally occurring transmission-reducing immunity. Specifically, antibody mediated transmission reducing immunity can be present in the absence or presence of antibodies specific to Pfs230 or Pfs48/45. Our work in **chapter 12** makes important steps toward understanding the mechanisms of transmission reducing immunity, by definitively proving that antibodies acquired naturally to Pfs48/45 and Pfs230 can contribute to the transmission blocking phenotype. We also strongly indicate the contribution of antibodies specific to unknown proteins, by showing that sera depleted of antibodies to key transmission blocking epitopes of Pfs230 and Pfs48/45 retained strong antibody mediated TRA. Importantly, we show that antibody responses to other gametocyte proteins bind to the surface of gametes, and are associated with TRA; the latter demonstrated by their association with reduced infectivity in both the SMFA with *Anopheles stephensi* mosquitoes and direct membrane feeding assay (DMFA) with *Anopheles gambiae* mosquitoes.

Malaria transmission reduction could be expedited by interventions targeted to the infectious reservoir of parasites or the deployment of drugs and vaccines that reduce human infectiousness. The emergence of parasite strains resistant to the best available anti-malarial drugs makes it urgent that we are better able to detect and deplete the malaria infectious reservoir, to prevent the spread of parasites that threaten malaria control globally.

Overzicht

Malaria blijft een ziekte met een enorme ziektelast. Met effectieve medicatie en de bestrijding van malariamuggen kunnen we het risico op (ernstige) ziekte voorkomen. Pogingen om malaria uit te roeien hebben baat bij een beter inzicht in het infectieuze reservoir van malaria; dat wil zeggen: de mensen binnen een populatie die hun infectie kunnen doorgeven aan muggen. Transmissiereducerende geneesmiddelen en vaccins hebben mogelijk een belangrijke rol bij malaria-eliminatie. Het vertalen van hun succes in het laboratorium naar toepassing in het veld vereist methoden om de infectiviteit van *Plasmodium* naar muggen te meten.

In hoofdstuk 2 hebben we een operationeel aantrekkelijke methode ontwikkeld om haarden van malariatransmissie te identificeren, gebaseerd op het aantonen van historische of huidige malaria-infecties. We hebben laten zien dat *Plasmodium* -DNA en menselijke, voor de parasiet specifieke, antistoffen tegelijkertijd kunnen worden geëxtraheerd en geanalyseerd. Deze methode is in het bijzonder geschikt voor het kwantificeren van malariatransmissie op populatieniveau. Op deze wijze kan de methode gebruikt worden voor de identificatie van gebieden met een hoge malariablootstelling waar gerichte interventies kunnen worden ingezet met als doel de transmissie te remmen en te voorkomen dat muggen worden geïnfecteerd.

Of muggen geïnfecteerd raken wanneer ze zich voeden op een geïnfecteerde gastheer hangt af van een reeks factoren waaronder gametocytdichtheid, de rijpheid van gametocyten, parasitaire stammen en menselijke immuniteit. Voedingsexperimenten waarbij muggen onderzocht worden na het nemen van een bloedmaal, vormen de enige betrouwbare methoden om te bepalen of een gastheer in staat is om muggen succesvol te infecteren en in welk percentage. Ons werk in **hoofdstuk 4** geeft een overzicht van de verschillende studies die het infectieuze reservoir direct hebben gekarakteriseerd met behulp van voedingsexperimenten. Wij zijn tot de conclusie gekomen dat dergelijke beoordelingen niet louter kunnen worden gebaseerd op voedingsexperimenten uitgevoerd in dwarsdoorsneden van populaties waar malaria voorkomt, maar een zo natuurlijk mogelijke weerspiegeling van variatie in blootstelling aan muggen moet bevatten. Deze muggenblootstelling bepaalt in hoge mate het transmissiepotentieel van individuen binnen het infectieuze reservoir.

Het begrijpen van vectorblootstelling is ingewikkeld, maar het kan eenvoudiger worden gemaakt door vectorblootstelling serologisch te bepalen. Ons werk in **hoofdstuk 3** was het eerste om de bruikbaarheid van antilichaamreacties op speekseleiwit (gSG6) van een malariamug te onderzoeken als een mogelijke indicator van heterogeniteit in vectorblootstelling. Een analyse zoals deze zou, in combinatie met infectiviteitsgegevens van voedingsexperimenten, kunnen worden gebruikt om heterogeniteit in transmissie op populatieniveau te beschrijven.

Belangrijke barrières voor een breder gebruik van voedingsexperimenten zijn de tijd en vaardigheid die het kost om de experimenten betrouwbaar uit te voeren. De grootste uitdaging hierbij is het aantonen van infecties in de muggen die gebruikt zijn bij de experimenten. In **hoofdstuk 7 en 8,** valideerden wij het gebruik van oocyst-prevalentie als een betrouwbare proxy voor het infectieus worden van muggen en vergeleken methoden die het mogelijk maken muggenbesmetting snel aan te tonen in voedingsexperimenten. Deze voedingsexperimenten zijn essentieel voor de evaluatie van op transmissie gerichte interventies; de ontwikkeling van deze interventies begint in het laboratorium. In **hoofdstuk 5**, ontwikkelden we een variant op het standaard membraanvoedingsexperiment (*standard membrane feeding assay;* SMFA) dat grotere schaalbaarheid heeft door gebruikmaking van een objectief, op luminescentie gebaseerd eindpunt voor de beoordeling van het aantal muggeninfecties en de analyse van transmissiereducerende activiteit (TRA). Het gebruik van transgene parasieten zorgt ervoor dat dissectie van muggen niet meer nodig is. In **hoofdstuk 6** werd de luminescentie SMFA aangepast voor gebruik met een in ons laboratorium ontwikkelde parasietlijn, die geschikt is voor de screening van geneesmiddelen. We hebben aangetoond dat bepaalde medicijnen werkzame eigenschappen hebben, die niet eenvoudig kunnen worden gedetecteerd door *in vitro* testen van gametocyt dodende activiteit. De SMFA blijft bij geneesmiddelenevaluatie hiermee noodzakelijk.

De latere hoofdstukken van dit proefschrift hadden als doel het menselijk infectievermogen te ontleden door rekening te houden met parasitaire en menselijke factoren die samen invloed hebben op de kans dat muggen besmet raken als ze malariageïnfecteerde mensen steken. De voornaamste factor hierbij is de gametocytdichtheid. Vanwege hun vaak zeer lage dichtheden is het vrijwel onmogelijk om door middel van microscopie het totale gametocytenreservoir te detecteren. Moleculaire testen laten zien dat infecties met submicroscopische gametocytdichtheden vaak infectieus zijn voor muggen; wiskundige modellen tonen daarnaast aan dat deze submicroscopische gametocytdichtheden relevant zijn voor de verspreiding van malaria op populatieniveau. In **hoofdstuk 9** en **10** gebruikten we gezuiverde transgene mannelijke en vrouwelijke gametocyten om een nieuwe kwantitatieve methode voor gametocyten te ontwikkelen, die in staat is de dichtheid van zowel mannelijke als vrouwelijke gametocyten te bepalen over het gehele biologisch plausibele bereik van infectiviteit. In **hoofdstuk 9** hebben we aangetoond dat een nauwkeurig vastgestelde gametocytdichtheid sterk geassocieerd is met de kans dat muggen besmet raken en dat de geslachtsratio een relevante invloed heeft op deze associatie. In **hoofdstuk 10**, gebruikten we dezelfde methode om het effect van een gametocyt dodend geneesmiddel, primaquine, te onderzoeken in een klinisch onderzoek in Keniaanse kinderen. De moleculaire methoden die zijn beschreven in **hoofdstuk 9 en 10** maken het mogelijk gametocytdichtheid, geslacht en infectiviteit in groot detail te bepalen.

Naast gametocytdichtheid en -rijpheid, kunnen menselijke immuunreacties een doorslaggevende factor zijn bij malaria-overdracht naar muggen. De bepaling van

menselijke immuunreacties die de transmissie van malaria van mens naar mug remmen is van zeer groot belang bij de evaluatie van transmissieblokkerende vaccins. Zoals uitgebreid besproken in **hoofdstuk 11,** kunnen antistofreacties tegen de gametocyten / gameet-eiwitten Pfs48 / 45 en Pfs230 slechts ten dele natuurlijke transmissiereducerende immuniteit verklaren. Antistof-gemedieerde transmissiereducerende immuniteit kan aanwezig zijn in de afwezigheid of aanwezigheid van antistoffen die specifiek zijn voor de eiwitten Pfs230 of Pfs48 / 45. Ons werk in **hoofdstuk 12** boekt een belangrijke stap voorwaarts in het begrijpen van de mechanismen van de transmissiereducerende immuniteit. In dit werk leveren we definitief bewijs dat antistoffen voor Pfs48 / 45 en Pfs230, ontwikkeld na natuurlijke malariablootstelling, kunnen bijdragen aan het transmissieblokkerende fenotype. Ons werk levert sterke aanwijzingen op dat ook antistoffen die specifiek zijn voor onbekende eiwitten een rol spelen bij transmissiereducerende immuniteit. We toonden aan dat sera waaruit antistoffen tegen de belangrijkste epitopen van Pfs230 en Pfs48 / 45 waren verwijderd, sterke transmissie-reducerende activiteit behielden. We toonden bovendien aan dat onbekende gametocyteiwitten herkend werden en dat deze herkenning geassocieerd was met functionele transmissiereducerende immuniteit, zowel in de SMFA als in membraanvoeding experimenten die uitgevoerd werden tijdens natuurlijk opgelopen malaria-infecties.

Het terugdringen van de verspreiding van malaria kan versneld worden door interventies die specifiek gericht zijn op het infectieuze reservoir van malaria parasieten; bijvoorbeeld door de inzet van medicijnen of vaccins die de menselijke infectiviteit verminderen. De opkomst van parasitaire stammen die resistent zijn tegen de beste beschikbare antimalariamiddelen, maakt het dringend noodzakelijk dat we het infectieuze reservoir voor malaria beter kunnen detecteren en bestrijden, om op deze manier de verspreiding van resistente parasieten te voorkomen die de malariacontrole wereldwijd bedreigen.

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*Shared authorship

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Acknowledgments

Teun – you've been an excellent supervisor and (believe it or not) role model: perhaps not for the amount of coffee you drink, or the speed your hair has greyed since we met, but in most other aspects of your career. You set a high bar but trying to reach it has made me a better scientist, and a very different person to the ~~1~~223 year old that came to Radboud with you in 2012. Thanks for your ability to turn around drafts within days or hours, your high standards, your friendship, humour, and your trust. I owe you a huge amount, and hope we'll continue working together.

Thank you Robert, for your supervision, good humour, and your no-nonsense commentary during meetings and work discussions – equal parts useful and entertaining. I've really enjoyed my time in the department.

Thanks too, Chris. Your teaching during my masters is a big part of the reason I stayed on, and your supervision in London has been invaluable.

Thank you…

Amrish, for teaching me the tricks of the trade in Kenya, for your friendship there and in Nijmegen, and for letting me stay in your cupboard (twice!) when I didn't have a home. Also, for all the whisky.

Kjerstin, for your unmatched skill and incredible work ethic, for the teaching, and for making me want to travel again, I owe you a lot more than an acknowledgment.

Wouter, for your friendship, and your enthusiasm and positivity in the face of many days we spent homogenising mosquitoes.

Matthijs, for your 11th hour and utterly essential contribution to the transmission-blocking immunity project, for many useful conversations, and with a view to our continuing collaboration.

Sanna, for your friendship and collaboration on our review and with the DAPPI project.

Sophie J, for teaching me how to hold a pipette, and for your friendship since then.

Maarten, for the teamwork in the first half of my time in Nijmegen, the many hours we spent grinding and dissecting, and for introducing me to The National.

Geert-Jan, for your mosquito expertise, for teaching me the value of scientific rigour, and for your tireless enthusiasm.

Lisette, for your friendship, and our ongoing collaboration in Nijmegen.

Marga and Rianne, for your essential help and advice with culture, feeding, and microscopy.

Bronner and Lynn, for your assistance and encouragement.

Koen, Martijn and Karin, for letting me work with you and your parasite.

Patrick, for allowing me to work with you in Mbita, for introducing me to your excellent cow, and for putting up with my nagging.

Robin, Maureen, Paul, David, Albert, George, Selphas, and Nick, for your tireless work in Mbita. And Addah – for the best Githeri in Homa Bay county.

Jacqueline, Laura, Astrid and Jolanda, for all the mosquitoes, and for your impeccable taste in music.

Will R, for your help and advice over the years.

Karina, Ben, Rob, Else, Guido, Krystelle, Lino, Chiara, Ivo, Lotus, Shehu, Amanda, Roos, and everyone else I worked alongside at Nijmegen.

Helmi, for your friendship and council. I hope your PhD project may be a success, despite the challenges you've experienced.

Fitsum and Issaie, for several excellent and drunken Ethiopian meals.

Taco and Charlotte, for the P. berghei work related to chapter 6 – which will hopefully be continued.

Annemieke and Daphne, for your assistance behind the scenes, good humour, and for putting up with me in your office when I had nowhere else to sit.

Pietro Alano and Matt Marti, for your important contributions to the gametocyte assay and transmission-blocking immunity projects, respectivley.

Elke, for your assistance in Kenya, and for making it fun even when the project was less so.

Aaron, Simon, Menno, and the CDC team, for our collaboration in Kisumu.

Jenny, for letting me stay at your place in Kisumu while we worked together on the REDHOT project.

Phil and Jiin, for the excellent food, the tour of So Cal, and for many great project discussions at UCI.

Josh, for your company and humour in Irvine.

Sanjai, Bryan, and your team at the FDA, for our collaboration with the Malaria Vaccine Initiative.

Stefan and Ashley, and your teams in Washington, for allowing us to use your parasite, and for the collaboration that followed.

Isabelle, Sandrine, and your team at Institute Pasteur in Yaoundé and IRD Montpelier, for your invaluable samples and for your collaboration on numerous projects.

Merribeth, Emily, Rick, Franck, and all at the Malaria Vaccine Initiative, for your invaluable help with several useful manuscripts, and for your assistance with ongoing projects.

Joe, Andy, Doug, Jozelyn, Adam, Xiaowu and Angela, and the whole team at ADI, for letting me stay with you while I completed the microarray probing in Irvine, for teaching me the analysis, and for your ongoing co-operation.

Tom and Anais, for your collaboration and statistical assistance with various projects.

Kevin, for many useful conversations at LSHTM, and for your collaboration with SIGNAL part 2.

Tate, Tom, Gillian, and all other members of the Drakeley group at LSHTM, for many useful work meetings.

Amy and Brandy, for your friendship in Kenya. And Rob, for your beard; a constant source of inspiration.

Anja and Daniel, for your friendship and advice in the latter half of my time in Nijmegen.

Sarah M, for letting me stay on your floor, for enduring my phlegmatism, for introducing me to the concept of breakfast, and for your friendship - which I have valued greatly.

Anna, Sarah F, Celia, Quique, Sophie, Steve, Minh, Duy, and little Minh-Anh, for your friendship, for putting up with my presence in group photos, and for taking the hat game so firmly to your hearts.

Manuella and Maggie for your friendship in and out of Kenya.

Finally...

Thank you to the volunteers in the Gambia, Cameroon, Uganda, Tanzania, Kenya, Burkina Faso, Mali, Britain, and the Netherlands who donated your blood so I and others could analyse it – some of you I met, most of you I didn't; this *couldn't* have been done without you.

Curriculum vitae

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Will Stone (1988) graduated from the London School of Hygiene and Tropical Medicine (LSHTM) with distinction in 2011. He worked for LSHTM in Kenya for 4 months, then moved to the Nijmegen, the Netherlands, to begin his PhD in Robert Sauerwein and Teun Bousema's group at the Medical Microbiology department of Radboud UMC. He has continued to work with Radboud UMC and with LSHTM, and is now based in London where he will begin his post-doctoral research into malaria transmission biology.

Education

2012-present Radboud University Medical Center, Nijmegen, The Netherlands
PhD Candidate, Supervisors: Teun Bousema, Robert Sauerwein, Chris Drakeley
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2010-2011 London School of Hygiene and Tropical Medicine, London, UK
MSc Medical parasitology
Grade awarded: Distinction (95.2%)

2006-2009 University of Bristol, Bristol & Avon, UK
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A/AS-Level: Biology (A), Geography (A), Chemistry (B), Art (A)
GCSE: 1 (A*), 8 (A), 1 (B)

Employment

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Researcher

2011 (Aug-Dec) London School of Hygiene and Tropical Medicine, London, UK
Research assistant, KEMRI/CDC Kisumu, Kenya; three months.

2010 (Jan-Aug) Brooms Barn Applied Crop Sciences, Rothamstead Research, Suffolk, UK
Field assistant; four months.

Publications

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Experience and skills

MSc	*Modules:*	Parasitology and Entomology (86.2%) Advanced diagnostic parasitology (80%) Immunology of parasitic infection: Principles (100%) Epidemiology and control of malaria (80%) Molecular biology and recombinant DNA techniques (100%) Advanced training in molecular biology (100%) Vector-parasite interactions (100%).
	Dissertation:	Epidemiological utility of Anopheles gambiae salivary antigen gSG6 (100%)
	Award:	Awarded the 2011 Patrick Buxton Memorial Medal and Prize
PhD	*Short courses:*	Advanced course in epidemiological analysis, LSHTM (2012) Pathogen genomics and Genomic epidemiology, LSHTM (2013)
	Presentations:	Annual conference of the American Society of Tropical Meicine and Hygiene (ASTMH), Atlanta, USA (2012) *(Oral)*, Uniting streams, Amsterdam, Netherlands (2012) *(Oral)*, LSHTM Malaria centre retreat, Cambridge, UK (2012, 2015, 2016) *(Oral, Poster)*, TransEPI, Barcelona, Spain (2013) *(Oral, meeting)*, ASTMH, New Orleans, USA (2014) *(Poster)*, ASTMH, Philadelphia, USA (2015) *(Oral)*, NVP Dutch malaria day, Leiden, Netherlands (2015) *(Oral)*
	Training:	Teaching assistant, Radboud Institute for Health Sciences, Vaccine Development Module (2015) Facilitator and speaker at Malaria Vaccine Inititative pan-African direct membrane feeding assay workshop in Yaounde, Cameroon (2014) Training and co-supervision of two students, and numerous lab staff in Mbita, Kenya during epidemiological (2013) and clinical trials (2015). Co-management of clinical trial in Mbita, Kenya (Dihydroartemisinin-piperaquine With Low Dose Primaquine to Reduce Malaria Transmission (DAPPI), Clinicaltrials.gov Identifier: NCT02259426)

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